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CONTRACT NO: DAMD17-92-C-2504

TITLE: SUPPORT FOR HIV-1 INTERVENTION THERAPY

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REPORT DATE: October 1, 1993

TYPE OF REPORT: Annual Report

PREPARED FOR: Commander
U.S. Army Medical Research, Development,
Acquisition and Logistics Command
(Provisional), ATTN: SGRD-RMI-S
Fort Detrick, Frederick, MD 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1 October 1993	Annual Report (9/30/92-9/29/93)

4. TITLE AND SUBTITLE
Support for HIV-1 Intervention Therapy

5. FUNDING NUMBERS
Contract No.
DAMD17-92-C-2504

6. AUTHOR(S)
Owen S. Weislow

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
SRA Technologies, Inc.
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research, Development,
Acquisition and Logistics Command
(Provisional), ATTN: SGRD-RMI-S
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This contract is intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. The primary objectives of the contract as stipulated in the contract award, are (a) to develop assays to evaluate the viral and immune responses to anti-retroviral therapy to include neutralization assays and drug susceptibility assays using clinical HIV isolates, (b) to develop and validate assays that predict or demonstrate disease progression for use in interventional trials with an emphasis on molecular biologic approaches to viral characterization and quantification of viral burden, and (c) to improve the accuracy, reliability, and cost effectiveness of clinical laboratory tests for all stages of HIV-1 and other retroviral infections. The contractor has incorporated two working groups to develop and optimize the necessary assays in support of these clinical studies and to establish production-level protocols.

14. SUBJECT TERMS
HIV, Therapy, RAD I, HIV-1

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Contract DAMD17-92-C-2504, Support of HIV interventional trials, was initiated in October of 1992 and is administered by Dr. Owen S. Weislow of SRA Technologies, Inc.. This contract is intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. The primary objectives of the contract, as stipulated in the contract award, are a) to develop assays to evaluate the viral and immune responses to anti-retroviral therapy to include neutralization assays and drug susceptibility assays using clinical HIV isolates, b) to develop and validate assays that predict or demonstrate disease progression for use in interventional trials with an emphasis on molecular biologic approaches to viral characterization and quantification of viral burden, and c) to improve the accuracy, reliability, and cost effectiveness of clinical laboratory tests for all stages of HIV-1 and other retroviral infections. The contractor has, in consultation with the contract office's representative (Dr. Douglas L. Mayers, Capt, USN), incorporated two working groups to develop and optimize the necessary assays in support of these clinical studies and to establish production-level protocols. The Two working groups include the Molecular Biology Working Group and the Cellular Phenotype Working Group. The work scopes of each are briefly described below with a detailed discussion of the progress made by each section. A Third section, the Data Group Section, consisting of computer personnel from SRA, has been established to support the efforts of SRA to improve delivery of all necessary data elements to WRAIR personnel as stipulated in the contract award.

1. Molecular Biology Working Group

During the first year (FY 1993) of this contract, there were three major areas of development conducted in the Molecular Biology section. These were the development of a quantitative RNA PCR methodology, development of an improved PCR assay for the presence of AZT-resistance associated mutations at HIV-1 Reverse Transcriptase amino acid's 215 position, and the development of diagnostic DNA sequencing protocols for use in support of drug-resistance monitoring for clinical trials. Information on the rational behind these protocols as well as background information will be provided below, along with a summary of the development of each of these methodologies, followed by copies of the current protocols for each.

a. Quantitative RNA PCR Protocol Development

The appearance of mutations in plasma prior to the detection of the

same mutations in patient cells[1] suggests that the source of plasma free virus is other than circulating PBMC. Thus, analysis of plasma viremia, both quantitatively (i.e., virions/ml) and qualitatively (i.e., mutation analysis), constitutes an informative measurement for clinical trials. One of the best ways to monitor this information is through the use of the polymerase chain reaction[2-4]. This technique was applied early on for HIV detection [5,6]. Amplification by PCR can allow detection of the low levels of retroviral nucleic acids in patient specimens, which are difficult if not impossible to detect by conventional methods (Southern, Northern blots) in most patients. Stimulated by the revolution in molecular biology caused by PCR, other amplification techniques, both target- and probe-based, have been described, and a plethora of new techniques for analysis of nucleic acids, based both on size fractionation (new gel techniques, anion-exchange HPLC, capillary zone electrophoresis), and hybridization analysis (greater availability of high specific activity enzyme-linked probes, fluorescent and chemiluminescent probes), have been described. SRA Technologies has developed expertise with several of these methodologies, and after careful evaluation of several of these technologies, has chosen a microtiter-based quantitation method for application to clinical studies support for WRAIR under this contract.

Quality Control for Quantitative PCR

The exquisite sensitivity of PCR, coupled with its ability to amplify DNA sequences many million fold, can potentially lead to false positive reactions due to contamination with amplifiable DNA not derived from the specimens to be tested. Such contaminating DNA generally originates from two possible sources: native DNA template introduced through specimen cross-contamination, or carryover from previous amplification reactions. SRA has applied several years experience using PCR for diagnostic testing to this problem and has considered the following factors in its protocol design.

The use of probe hybridization or size fractionation by electrophoresis or HPLC for post-PCR analysis allows one to increase the target-derived sequence, but not the assay background, by simply increasing the number of cycles. However, high-sensitivity amplifications are more susceptible to product carryover contamination, and it is essential to take strong precautions against PCR product carryover. It should be noted that amplification of low copy number targets may yield signals which are indistinguishable from low amounts of product carry-over. The importance of preventing product carryover in the context of

reliable detection of low copy number sequences cannot be overemphasized. With this need recognized, a number of techniques have been applied to reducing or preventing PCR-product carryover[7-9]. The application of one of these, uracil-n-glycosylase[9], in all of our PCR protocols to help reduce the chance of inaccuracy of quantitative determinations due to the presence of contaminating DNA.

In the course of our work for the Department of Retrovirology, we have evaluated UV irradiation, isopsoralen inactivation of PCR products, and the use of UNG; in our experience, UNG is both more effective at preventing PCR product carryover, and easier to implement uniformly in the laboratory for both detection and quantitation protocols. In this method, dUTP substitutes for dTTP in all PCR reactions and UNG is included in all reactions. Prior to the start of temperature cycling, this moderately heat stable enzyme selectively excises all uracil residues present in the DNA. DNA strands containing the resulting apyrimidinic sites are broken during the initial heating step, thereby preventing their functioning as templates in subsequent PCR cycles. Since only DNA from previous PCR reactions would contain these templates, the actual specimen DNA to be tested is unaffected. The *Taq* DNA polymerase incorporates dUTP as efficiently as the normal dTTP during PCR, so this technique can be used without decreasing the sensitivity or specificity of PCR reactions. Use of dUTP and of UNG in all PCR reactions eliminates the most common cause of a false positive result--carryover of amplified DNA from a previous amplification. SRA has incorporated this technique into PCR protocols for the amplification of HIV-1/2 and HTLV-I/II, and into quantitative PCR for viral DNA.

In addition, all reagents are prepared as single-use aliquots, tested for contamination and PCR performance, and then frozen for future use. Sample preparation, PCR reaction setup, and PCR product analysis are all performed in separate rooms with dedicated equipment, including certified Biosafety cabinets equipped with HEPA filters for all steps involving potentially infectious materials. For all pipetting steps prior to specimen amplification, either positive displacement pipettors with single-use tips and pistons or special aerosol-barrier pipet tips are used.

A negative control for sample preparation is included with each panel of specimens tested. Negative controls for contamination during reaction set-up consist of DNA from specimen known to be negative for the sequences being amplified. These negative controls are prepared and inserted between each set of duplicate reactions. This proximity of negative controls to specimen reactions is essential to

evaluate the possibility of contamination arising from handling errors and/or aerosol generation during reaction preparation. Such controls are essential when quantitation of low copy number targets is required.

In opposition to the potential for false positive reactions, or increased signal due to amplification of contaminating DNA, the sequence variation in HIV necessitates careful primer design to ensure detection of varied isolates and the elimination of false negatives. If diagnostic or quantitative PCR is to be applied to isolates which have been either sequenced or tested with multiple PCR primer pairs (e.g., in the anchored PCR system), a more informed choice can be made with respect to primer sets which are likely to amplify efficiently. Based on both literature review and information obtained from Dr. Francine McCutchan (personal communication), we have chosen primers for quantitative PCR that reside in conserved regions of the HIV-1 GAG gene to provide the greatest potential for amplification from all patient isolates.

In addition to the attention given primer design, all other aspects of the PCR protocols are carefully optimized for both specificity and product yield. These procedures include empirical determination of optimal oligonucleotide ratios and concentrations, magnesium ion concentration, *Taq* polymerase concentration, and annealing (hybridization) temperature for each primer set employed. Despite these precautions, some non-specific annealing of PCR primers does occur, particularly with PCR protocols such as those required for retroviral detection, where the target sequence must be amplified in the presence of a high background of genomic DNA. While post-PCR hybridization detection ensures that non-specific products will not be detected, the unintentional synthesis of these amplicons can affect the amplification process overall. If non-specific products are produced during the early cycles of the PCR process, they serve as templates for the synthesis of large quantities of DNA. This detracts from the overall efficiency of the reaction, as both specific and non-specific products compete for primer binding and *Taq* polymerase extension. Careful adjustment of reaction conditions to strike a balance between maximization of primer hybridization and minimization of non-specific annealing can significantly increase PCR product yield and also extend sensitivity into the sub-10 copy range. In our experience, with some primer sets, the detection limit can be extended by as much as 2 to 3 orders of magnitude using these optimization strategies. Rapid PCR product analysis by HPLC is particularly useful for developing new protocols, often allowing complete optimization of reaction conditions for a new primer set within a few days.

The use of a Hot Start[10,11] PCR protocol, recently aided by the introduction of AmpliWax™ (Perkin Elmer), can significantly increase the sensitivity of the PCR reaction. By preventing primer annealing at the non-stringent temperatures that occur at the start of the PCR reaction, prior to the first denaturation step, the production of non-specific DNA is minimized or prevented entirely. The elimination of these non-specific sequences leads to significant improvement in the yield of the desired reaction products, thereby increasing sensitivity. The Hot Start technique is performed by separating reaction components until stringent temperatures are attained. For example, if *Taq* polymerase is added only after reactions reach approximately 70-80°C, no products will be synthesized while the reactions are at non-stringent temperatures (room temperature up to the optimum annealing temperature of that primer set).

The use of AmpliWax™ greatly facilitates this procedure in the following manner. A single wax bead is placed into each reaction tube or well along with all the PCR reaction components except *Taq* polymerase and the specimen to be tested. The reactions are heated for 5 minutes to melt the wax and are then cooled to room temperature, allowing the wax to resolidify, creating an impermeable barrier. The sample to be tested is then added along with the *Taq* polymerase, and PCR conducted using the optimized conditions determined for that primer set. During the first PCR cycle, as the temperature rises through about 65°C to 80°C, the wax melts, allowing the reaction components to mix by convection, and PCR proceeds in the standard fashion. Increased specific product yield without non-specific product synthesis results in a more sensitive assay, which is even more apparent in terms of signal background in the microplate assay described in this section. In addition, the use of Hot Start also allows the use of an increased number of cells in the specimen sample, increasing the total quantity of target DNA present; with Hot Start, the inclusion of DNA from at least 1 X 10^6 cells is possible without adversely affecting the reaction efficiency. This advantage is significant in any PCR application requiring the detection of a low copy number target, such as HIV during early stages of infection, which may be present at low abundance in peripheral blood.

Accuracy and Precision of the Quantitative PCR Protocol

In analytical chemistry, accuracy is defined as the nearness between the measured value and the true population average. Precision is defined

as the degree of agreement between or among measurements. As applied to quantitative PCR protocols, it is essential to differentiate between accuracy and precision.

The error associated with the prediction of initial copy numbers from quantitative PCR data is associated with the error in measurement of product concentration. Thus, methods which allow precise measurement of PCR product will also be the methods which allow most precise extrapolation to initial template copies. We have characterized the precision of several techniques. Instrumental methods, which do not depend on technicians, are capable of highly precise measurements. For example, we[12] and others[13] have reported variation among replicate HPLC determinations of PCR products to be within $\pm$ 3-4%. However, we have also found that hybridization analysis by a number of methods is also capable of high precision, when properly designed, and when pipetting technique is carefully controlled. All technicians who perform the quantitative PCR protocol are thoroughly trained in the procedure and have extensive experience in it's use.

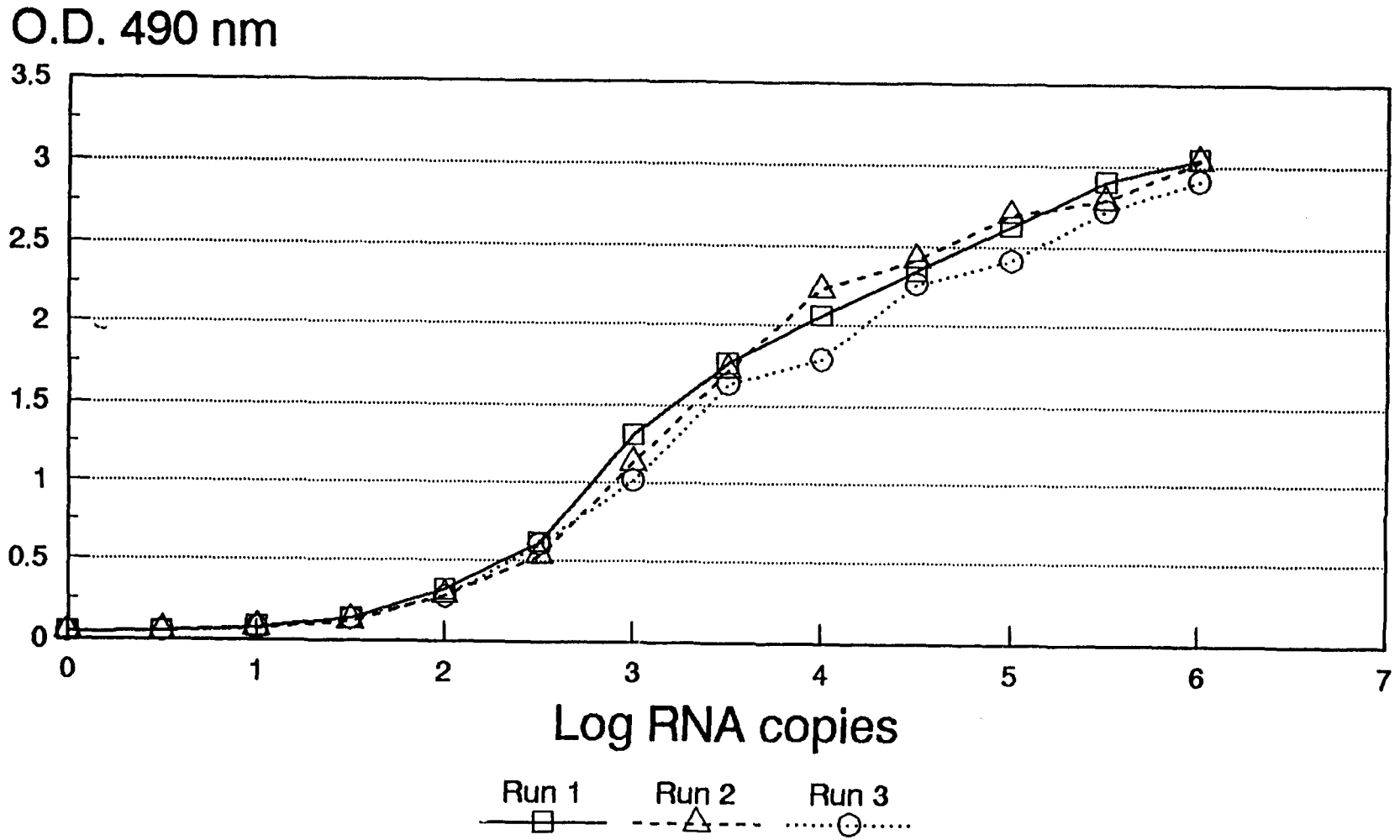
The frequent observation that biological assays are highly variable is probably true, but PCR and, particularly, post-PCR analysis, are not biological, but chemical, processes. Thus, if all variables are tightly controlled, good reproducibility will be obtained. We have characterized the variation associated with each part of the quantitative PCR (Figure 1; run-to-run variation for PCR and PCR product analysis: Figure 2; extraction-to-extraction variation), and strongly feel that the most significant variation is likely to enter at the level of nucleic acid extraction. When plasma RNA extract from an HIV-infected patient was amplified as three reactions in each of 3 separate RNA PCR runs, with analysis by capture plate, c.v. of 1.2%, 14.9%, and 12.5% were obtained for the three runs.

Extensive experimentation was also done to determine the best protocol for the actual extraction of viral RNA from plasma. A number of methods were chosen, with the best 3 protocols compared in table 1. As can be readily seen, extraction of the viral RNA with guanidinium produces the best yield of recovered RNA as evidenced by the higher figures in the last column. Based on these and similar results, we have chosen to use a guanidinium extraction of viral particles to liberate and preserve the viral RNA following centrifugation to collect the virus.

The variation of the entire PCR reaction is compared in Figure 1,

where three independent standard curves comprised of 5 replicate reactions at each copy number of an RNA transcript dilution series as follows: 3, 10, 30, 100...10^6/reaction. Variations among the 5 replicates ranged from $\pm$ 20.6% to $\pm$ 0.3%, with most c.v. < $\pm$ 5.0%. Amplification was for 30 cycles, in the Perkin Elmer 9600 cycler. Product analysis was by capture plate with HRP-linked colorimetric readout. Each plotted data point constitutes the mean of 5 replicate amplification reactions for each of the three runs. As can be readily seen, the shape of the curve is almost identical at all points. The position of the linear portion of the curve can be varied somewhat if needed (to encompass sample values of particularly high or low numbers) simply by varying the incubation time of the substrate with the enzyme (HRP)

Figure 1. Variation in Quantitative RNA PCR



PCR-3135, JW, 2/22/93
3 runs, 9600, 30 cycles, 0.2 uM primer GAG-3/6
capture assay with HRP-GPR-5

Figure 2. Run-to-Run Variation in Predicted Plasma HIV-1 RNA

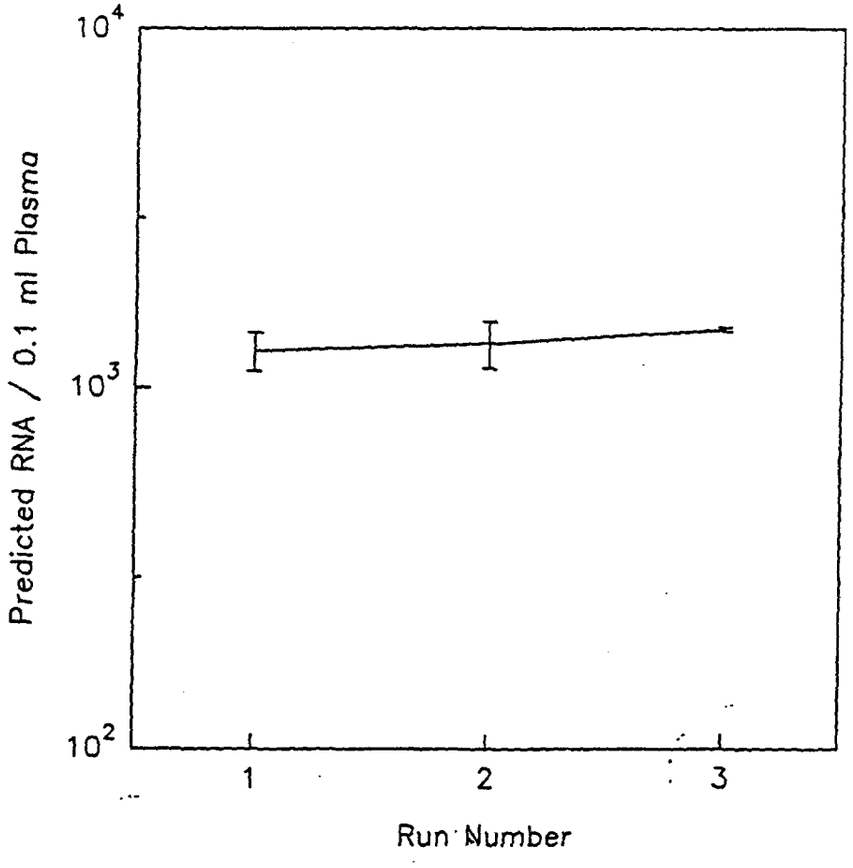


Table 1

COMPARISON OF RNA EXTRACTION TECHNIQUES ON PATIENT PLASMA

Method	rep.	mean O.D. ± s.d.	mean O.D. ± S.E.
crude lysis	1	0.358 ± 0.052	0.453 ± 0.040
	2	0.492 ± 0.204	
	3	0.402 ± 0.023	
	4	0.379 ± 0.013	
	5	0.590 ± 0.056	
	6	0.495 ± 0.061	
guanidinium	1	0.735 ± 0.056	0.710 ± 0.029
	2	0.792 ± 0.134	
	3	0.747 ± 0.059	
	4	0.718 ± 0.035	
	5	0.630 ± 0.024	
	6	0.635 ± 0.030	
proteinase k	1	0.095 ± 0.004	0.161 ± 0.030
	2	0.061 ± 0.006	
	3	0.206 ± 0.042	
	4	0.176 ± 0.029	
	5	0.220 ± 0.033	
	6	0.206 ± 0.078	

Patient plasma (0.5 ml) was ultracentrifuged to pellet virus. The viral pellet was then extracted by the indicated method. The crude lysis technique used an RNasin-containing 2.5% NP-40. Guanidinium/phenol used a one-step commercially available reagent. Proteinase k/phenol used proteinase k digestion, followed by two phenol extractions, according to a protocol received from GeneLabs. Amplification was for 30 cycles with the gag primers SK 38/39. Product quantitation was performed by the capture assay with HRP-linked probes.

Protocol for Quantitative PCR to Determine Plasma Viremia

Over the past year, a large amount of development work on this assay has allowed us to validate the various parts of the assay. The current protocol in use in our laboratory for the support of WRAIR contracts is given in the following sections.

The primer set[14,15] used in the procedure described below allows determination of HIV-1 genomic RNA. Specific amplification of unspliced transcripts is obtained by use of primers which bracket a splice junction.

Sample preparation from plasma by the protocol described here results in an RNA preparation which is substantially free of DNA. Amplification of extracts prepared by this procedure for 30 cycles do not result in detection of any specific signal in a "no-RT" reaction (F. White, M. Vahey, unpublished observations). Extraction by this procedure has been shown by us to give consistent yields (previously shown in Figure 2), comparing favorably with other techniques which we have tested.

Specificity for HIV-1 is ensured by the use of a specific oligo (i.e., the anti-sense PCR primer) to primer cDNA synthesis, optimized PCR conditions with specific primers, and hybridization analysis in a sensitive and precise capture assay.

Extraction of RNA from Plasma

Plasma or serum may be used as substrates for this assay, but it is anticipated that fresh (<8h post-draw), unfrozen plasma will allow more accurate determination of plasma viremia. Other biological fluids such as cerebrospinal fluid (CSF) could potentially be used. In all cases, the specimen, if plasma or serum, should be maintained at 4°C, not frozen, and delivered for assay as soon as possible following draw. If the specimen is whole unfractionated blood, it should be maintained at room temperature (20°C to 24°C) and should be delivered for assay as soon as possible following draw (within 8 h).

The meaningful use of PCR to estimate plasma viremia requires some knowledge of recovery during extraction. While recovery with a crude extract method for DNA preparation does not present any significant opportunity for DNA loss, RNA extraction involves many such opportunities. With PBMC's, a human marker gene can be amplified to probe whether significant RNA loss occurred during sample preparation. However, this cannot be done with plasma. Therefore, a known amount of non-HIV derived RNA transcript can be added, and a subsequent PCR carried out for these sequences for the purpose of confirming that no significant loss of RNA occurred during sample preparation. This method, however, provides no control for degradation of RNA through virus lysis prior to addition of guanidinium-phenol reagent.

If the sample is frozen, thaw by manual agitation in a 37°C water bath, then maintain on ice. In the case of whole blood, plasma is prepared according to SRA's SOP for this procedure, which is presented as follows, for clarity.

Preparation of Plasma from Whole Blood

1. Centrifuge the whole blood at approximately 200 X g (1400 rpm) in a tabletop centrifuge at 4°C.

2. Remove the supernatant, taking care not to disturb the cell layer.

3. Centrifuge the supernatant from Step 2 at approximately 1000 X g (4000 rpm) in a tabletop centrifuge at 4°C. The purpose of this step is clarification, to remove residual cells.

4. If the plasma is to be extracted immediately, maintain on ice. If it is to be stored, aliquot into 1.0 ml cryo-vials, and store at -70°C.

Pelleting of Virus

1. Add approximately 2 ml PBS/BSA (PBS containing 5 µg/ml BSA) to a Quick-Seal polyallomer ultracentrifuge tube. Alternately, a microfuge can be used to collect virus by pelleting. In this case, 2 volumes of PBS/BSA are added to 500 µl of plasma in a 2.0 ml screw-capped microfuge tube. Be sure to record the exact volume of plasma used at this stage if other than 500 µl.

2. Pellet the virus by ultracentrifugation in the 50.3Ti rotor at 40,000 rpm at 4°C for 1h, or similar run conditions in a similar rotor. If using a microfuge, spin at top speed (>10,000 xG) for 30 minutes at 4°C.

RNA Extraction

1. Remove the supernatant from the ultracentrifuge tube, transferring to a fresh, sterile microcentrifuge tube.

2. Add 800 µl Tri-Reagent (guanidinium/phenol). Vortex 15 s.

3. Allow to sit at least 5 min. at room temperature.

4. Add 160 µl $CHCl_3$ to each tube. Vortex 15 s.

5. Allow to sit at least 3 min. at room temperature.

6. Centrifuge at maximum speed (approximately 10,000 X g) in a microcentrifuge at 4°C for 3-5 min.

7. Remove the aqueous (upper, colorless) phase to a fresh tube.
8. Add 400 µl isopropanol (IPA, 2-propanol) and 5 µl of 2 µg/µl glycogen to each tube. Mix well by vortexing.

9. Maintain at -20°C overnight. Shorter times (between 1 hour and overnight) can be used will good, but less than optimal recovery of the RNA.

10. Centrifuge at maximum speed in a microfuge at 4°C for 30 min.

11. Decant the supernatant.

12. Wash the pellet with 1 ml of ice-cold 75% ethanol. Vortex briefly.

13. Centrifuge at maximum speed (approximately 10,000 X g) in a microfuge at 4°C for 30 min.

14. Decant the supernatant.

15. Air dry the pellet. Do not use a Speed-Vac.

16. Add Virus Lysis Buffer A (2.5% NP-40, 0.2 mg/ml tRNA, 1 U/µl RNasin, 2 mM DTT) for 10 µl per 0.1 ml original specimen volume. At least 50 µl of reagent should be used, regardless of specimen volume. Vortex.

17. Maintain at 42°C approximately 15 min. to fully dissolve the RNA, after which the extract should be stored at -70°C. The RNA should not be heated above 60°C until after reverse transcription, as RNasin in the Virus Lysis Buffer will be inactivated.

Quantitative RT/PCR with Genomic RNA Specific Primers

The following procedure supports amplification of genomic, unspliced HIV-1 RNA, with sensitivity to approximately single copy detection, when 36 cycles of amplification are used. Other reaction conditions (Mg^{2+} concentration, primer concentration, Units *Taq* polymerase per reaction, and annealing temperature) may

be required with other primer sets, in order to attain requisite sensitivity.

Reverse Transcription

1. Prepare an RT mixture as follows. This reaction mix should be prepared on the same day it is to be used, and should be maintained on ice, and reagents should be added in the order listed (i.e., RNasin must be added after DTT, and RT should not be added to an unbuffered solution).

   4.0 µl 5X RT buffer *
   0.8 µl dNTP working stock for RT (25 mM each dNTP)
   2.0 µl 100 mM DTT **
   2.5 µl 20 µM 5'-biotin-GAG-6 ([illegible]-sense primer)
   0.5 µl RNasin (40 Units/µl, Promega Biotech)
   0.2 µl MoMuLV-RT (200 Units/µl, Gibco/BRL)

   10.0 µl / reaction

   * 5X RT buffer is supplied with MoMuLV-RT by Gibco/BRL. Its composition is 375 mM KCl, 250 mM Tris-Cl, pH 8.3, 15 mM $MgCl_2$.

   ** 100 mM DTT is supplied with MoMuLV-RT by Gibco/BRL.

2. Add 10 µl of RT mixture to each tube.

3. (If TC-1 is to be used, add 70 µl of mineral oil; if 9600 is to be used, add no oil.)

4. Pipet 10 µl of the appropriate specimen RNA into each tube.

5. Heat as follows by manual transfer between dry blocks. Alternatively, water baths or cycler units may be used.

42°C	15 min.
99°C	5 min.

   Transfer directly from 99°C to from 0-4°C. This is most conveniently done by pre-cooling a heat block in an ice water bath. However, ice or ice water may also be used.

PCR Amplification

1. Prepare a PCR reaction mix as follows.

66.1 µl H_2O
8.0 µl 10X PCR buffer (no $MgCl_2$)
4.2 µl 25 mM $MgCl_2$
1.0 µl 20 µM GAG-3 (sense) primer
0.5 µl Taq DNA polymerase (5 Units/µl)
0.2 µl uracil-N-glycosylase (1 Unit/µl)

80.0 µl / reaction

2. Pipet 80 µl of the PCR reaction mix to each tube containing 20 µl of RT reaction product.

3. Cycle as follows.

(for 9600 in *bold*)

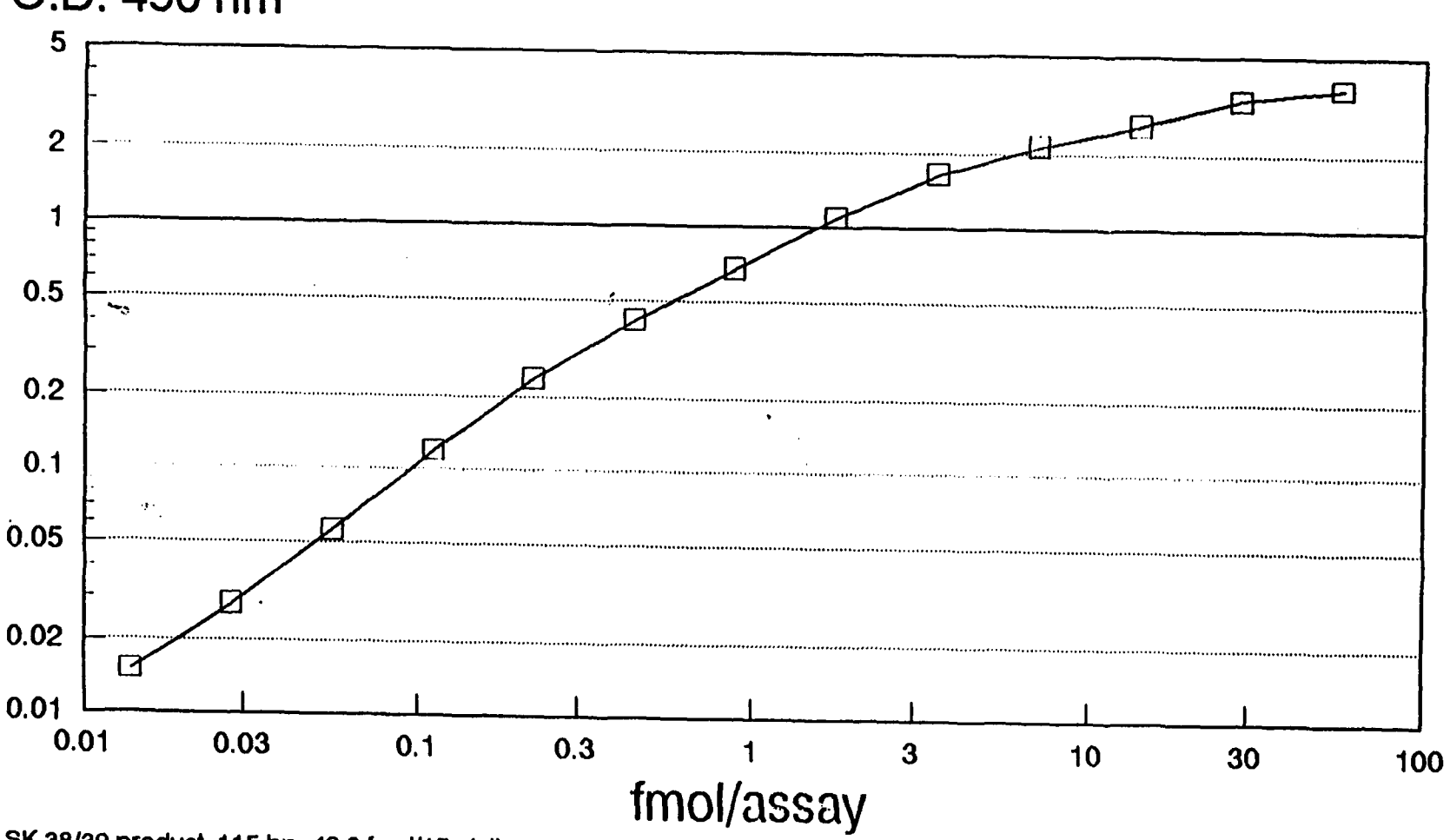
94°C	5 min.		*(5 min.)*	
94°C	1 min. 15 s		*(15 s)*	
55°C **(56°C)**	1 *min.* 15 s	*(30 s)*		
72°C	1 min.		*(30 s)*	X 10 cycles
92°C	1 min. 15 s		*(15 s)*	
55°C **(56°C)**	1 min. 15 s		*(30 s)*	
72°C	1 min.		*(30 s)*	X 26 cycles
72°C	maintain			

4. Following temperature cycling, maintain reaction products at 4°C no longer than 4 days. For longer storage, they should be maintained at -20°C or lower.

Quantitation of PCR Products by Affinity-Based Hybrid Capture

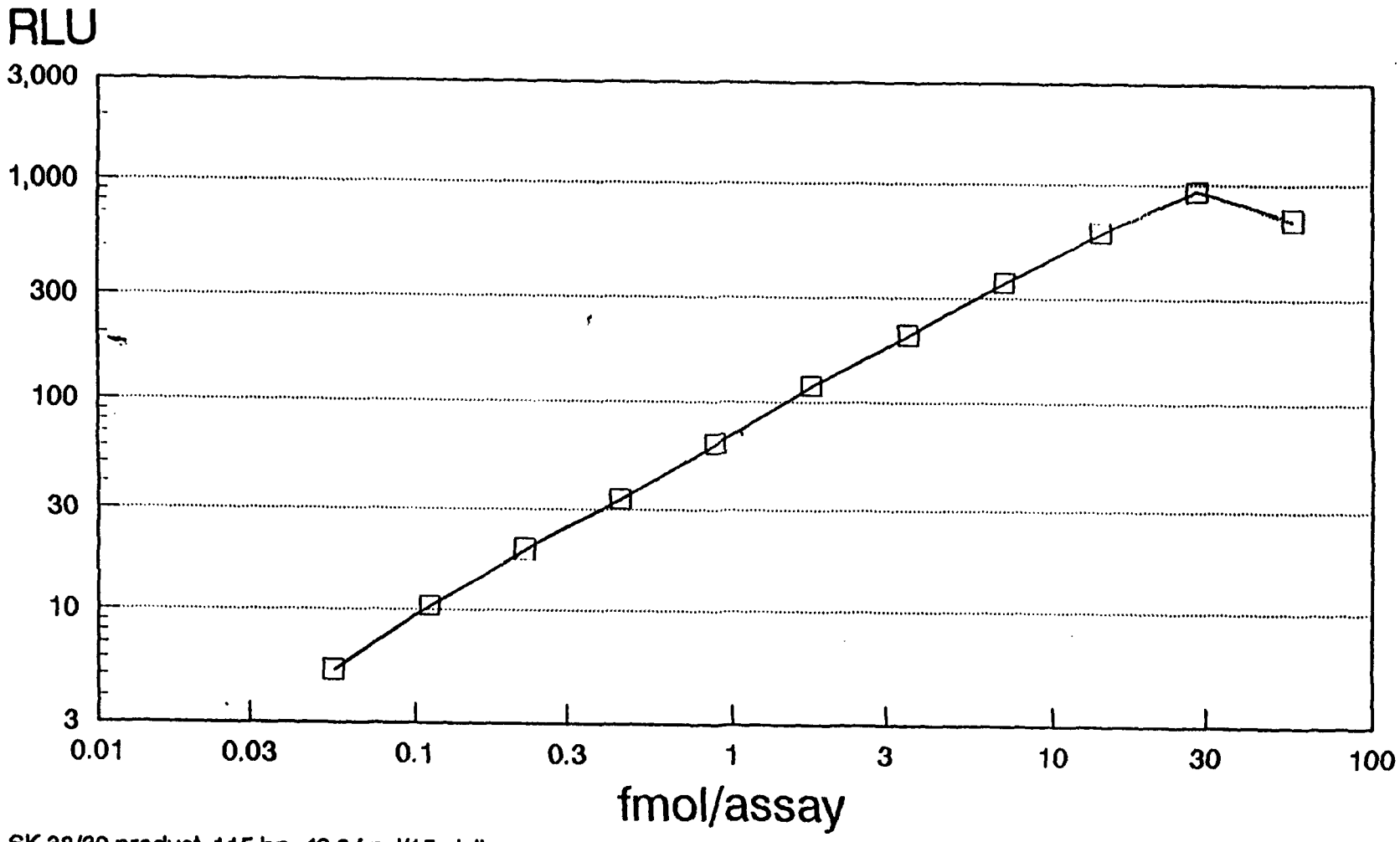
The following procedure allows precise quantitation of PCR products which have incorporated a 5'-biotinylated primer. Products are captured by means of the avidin-biotin interaction on the surface of a coated and blocked microwell. Products are then detected by hybridization to a direct enzyme-labelled oligo probe. We have used two different protocols over the past year for this detection scheme. One of

Figure 3. Sensitivity Comparisons: CP-HRP



SK 38/39 product, 115 bp, 42.3 fmol/15 ul, then diluted 1:2 with PCR mix
10 ul/well

Figure 4. Sensitivity Comparisons: CP-AP



SK 38/39 product, 115 bp, 42.3 fmol/15 ul, then
diluted 1:2 with PCR mix
10 ul/well

these utilizes alkaline phosphatase (AP) as the label linked to the probe oligo with detection using the chemiluminescent substrate LumiPhos (Lumigen, Inc., Detroit, MI). A similar assay, with colorimetric readout following the reaction of *o*-phenylenediamine (OPD) with a horseradish peroxidase linked probe, has been reported by Winters, et al[16], and has also been used in our laboratory. This protocol has some advantages over the chemiluminescent version in the areas of lower more consistent backgrounds and a slightly faster assay overall. Chemiluminescent detection, as described here, allows slightly wider dynamic range than colorimetric readout, but requires specialized instrumentation, such as a microplate luminometer. Additionally, the background of the assay is more variable than with HRP colorimetric detection. For use in less controlled or less technologically sophisticated settings, the colorimetric detection assay can be tuned to a wider or more narrow window by simply adjusting reaction times. Short reaction times give a useable dynamic range of approximately 100 copies to 10^5 copies, while longer times allow low copy sensitivity, but with O.D. reaching the maximum of the plate reader at high copy number. A comparison of standard curves generated using each of these protocols is given in Figures 3(HRP) and 4(AP). Both protocols are given in the following section.

Preparation of the Avidin Coated Microtiter plate

The preparation of the plate is identical for both AP and HRP linked detection systems.

1. Prepare an avidin-coated and blocked microwell plate according to the following procedure.

    a. Pipet 120 µl of 100 µg/ml avidin D (Vector Labs) into each well of a high-binding plate (e.g., MaxiSorp, Nunc; Immulon 4, Dynatech). Incubate overnight at ambient temperature.

    b. Remove the solution, and wash 4 times with Wash Buffer (1% Tween 20 in PBS).

    c. Pipet 200 µl of 1% casein (Hammarsten Grade, BDH) in PBS into each well. Incubate for 1 h to overnight at ambient temperature.

    d. Remove the solution. Store the plate frozen, under which conditions it remains stable for at least several weeks.

Analysis of PCR Products: AP Labeled Probe

1. Heat denature 10 µl of PCR products by incubation at 95°C for 5 min., followed by quick-cooling to approximately 4°C using an ice bath.

2. Pipet 90 µl of Hybridization Buffer (1% casein in PBS) containing 1 pmol of alkaline phosphatase-conjugated probe GPR-5 (Synthetic Genetics) into each coated and blocked well of a microwell plate.

3. Pipet 10 µl of PCR product into the appropriate well.

4. Incubate at 42°C for 20 min. to allow both hybridization and capture.

5. Remove the hybridization solution and discard. Wash the plate 4 times with Wash Buffer.

6. Pipet 100 µl LumiPhos into each well.

7. Incubate at 37°C for 30 min. Read immediately in the ML1000 microplate luminometer (Dynatech).

Analysis of PCR Products: HRP Labeled Probe

1. Heat denature 10 µl of PCR products by incubation at 95°C for 5 min., followed by quick-cooling to approximately 4°C using an ice bath.

2. Add 90 µl of HRP-probe (CRP-5 linked HRP) containing 1 pmol per 90 µl into each coated and blocked well of a microwell plate.

3. Pipet 10 µl of PCR product into the appropriate well.

4. Incubate at 42°C for 20 min. to allow both hybridization and capture.

5. Remove the hybridization solution and discard. Wash the plate 4 times with Wash Buffer.

6. Add 150 µl OPD substrate solution into each well.

7. Incubate at 25•C. for 10 min. NOTE: Time and temperature are critical. If ambient temperature is not 24-26•C., a temperature-controlled oven or incubator must be used. The time is measured from the first addition of OPD to the plate.

8. Stop the reaction by adding 100 µl 1 N H_2SO_4.

9. Read O.D. at 490 nm in a plate reader.

Additional development is planned for the quantitative protocol. This work includes evaluation of an automated nucleic acids extractor (Autogen 540) in comparison to the manual method, development of fluorescent substrates and readouts for the microplate assay, allowing increased sensitivity and greater dynamic range than either chemiluminescent or colorimetric assays, and the potential development and application of the NASBA™ (Nucleic Acid Based Amplification System) in collaboration with WRAIR and Organon-Teknika.

215 ARMS PCR Assay

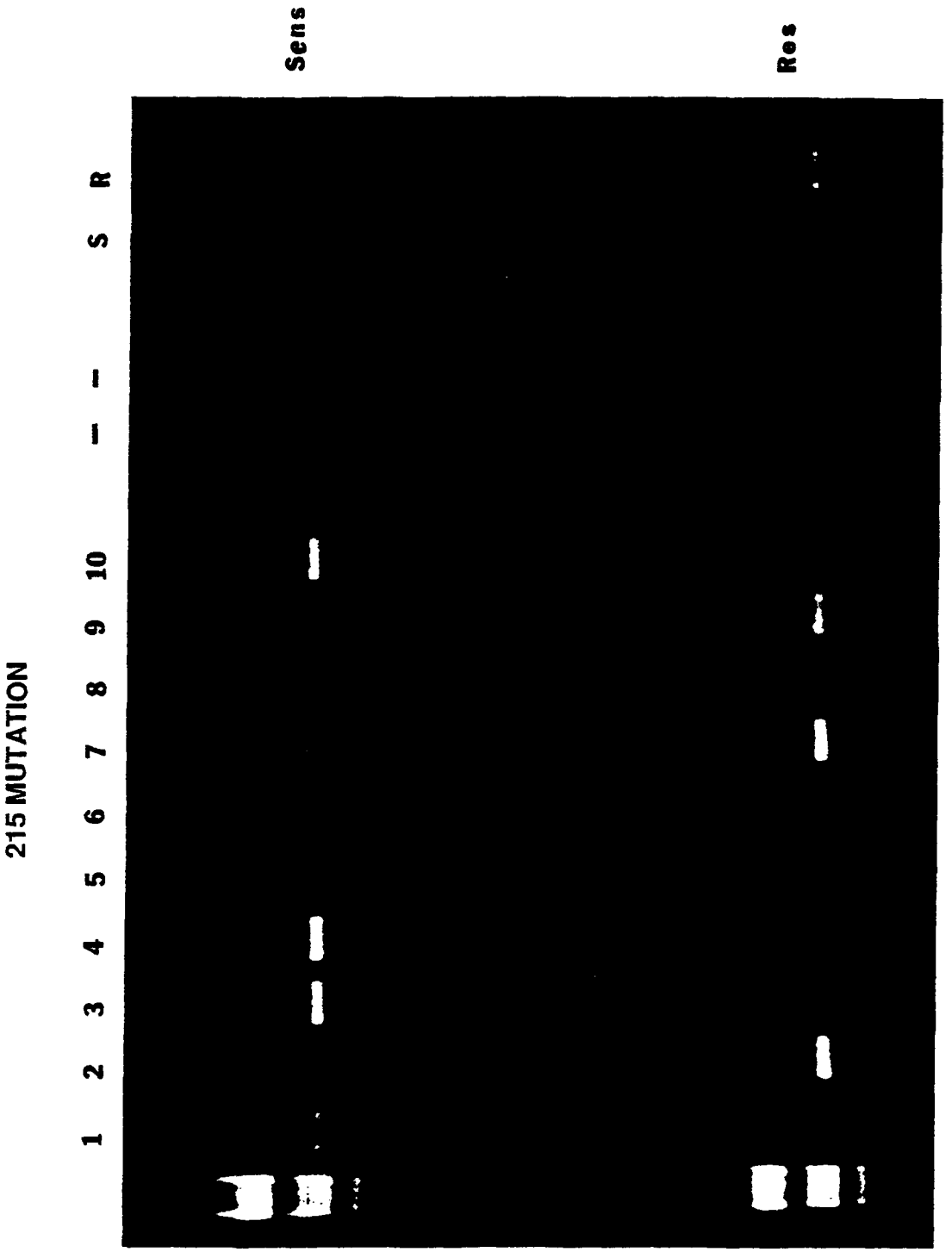
Of great importance in the type of WRAIR clinical trials supported by SRA Technologies is the monitoring of the acquisition of drug resistance. At this point in time, AZT (Zidovudine) is the primary drug used in the these trials. While potentially providing some benefit to the patient, the usefulness of AZT is limited by the tendency for almost all recipients to develop some level of resistance to the drug during the course of therapy. In order to better assess the effectiveness of treatment modalities, it is useful to have a rapid screening assay for patients that will indicate the onset of genotypic mutations associated with AZT resistance.

Amplification Refractory Mutation System

A combination of ASO (Allele Specific Oligonucleotide) techniques and PCR has been recently developed that makes use of the best aspects of both techniques. Variously named the Amplification Mutation Refractory System (ARMS) or PCR Amplification of Specific Alleles (PASA), this technique takes advantage of the inability of synthetic oligonucleotide primers that are incompletely hybridized to a template to serve as effective PCR primers[17]. First described by Markham et al. and Sommer et al.[18,19], this technique has been applied to the detection of single base changes and identification of specific alleles associated with disease in such diverse instances as cystic fibrosis[20,21], phenylketonuria[19], apolipoprotein genotyping[22], and HLA typing[23]. Larder et al. have recently applied this technique to examine AZT resistance acquired during chemotherapy, first, by characterizing the genetic mutations in the HIV RT gene that can be linked to in vitro resistance[24], and more recently by applying this technique to the direct determination of the presence or absence of these mutations in patient blood samples[25]. Further work by his group has validated and extended this approach [26-29].

Numerous mutations have been described within the HIV RT gene that confer in vitro resistance to both nucleoside and non-nucleoside RT inhibitors currently in use or under evaluation as potential HIV antiviral therapies. These include amino acids 67, 70, 215 and 219[24,26,30], and 41 for AZT[28], 181 for BI-RG-587 (Nevirapine)[31,32], and 69, 74, 184 and 294 for ddI (2',3'-dideoxyinosine) and ddC

Figure 5



(2',3'-dideoxycytidine)[26,33]. Many of these mutations are directly detectable using the ARMS technique.

In addition to the drugs currently available, a number of new agents are currently being developed and tested as HIV chemotherapeutic agents against both the HIV RT gene[34,35] and other viral targets such as the integrase protein[36], and the HIV protease[37]. It is expected that as the new agents and combination therapies are administered to patients, new mutations conferring resistance to these agents will also be discovered, and in fact, preliminary data indicates that is the case (Third Workshop on Viral Resistance, Gaithersburg MD, Sept 1993). It will be useful to monitor the appearance of resistant virus in patient populations in order to adjust the therapeutic regimes in use at the time. Although the details of the assay described below are for the detection of the mutation at amino acid 215 that confers AZT resistance, this procedure is readily adaptable to the detection and monitoring of mutations at other locations within the viral RT gene simply by changing the primers used in the second (nested) PCR reaction and re-optimizing the PCR reaction conditions (if needed) to maximize sensitivity.

SRA Technologies is currently applying this technique as an aid in assessing the development of AZT resistance in patients undergoing chemotherapy. We are also participating in the ongoing AIDS Clinical Trials Group (ACTG) 215 Working Group to improve and standardize the assay for general use as a diagnostic tool. Our current protocol for the detection of AZT resistant genotypes at amino acid 215 in the HIV RT gene is described below, and was recently presented at the Third Workshop on Viral Resistance in Sept. 1993. An example of the effectiveness of this protocol is seen in Figure 5. This exhibit shows a panel of 10 samples tested with both the wild type (sensitive) and mutant (resistant) primers as described in the following sections. 7 of 10 samples give a very clear result (the presence of a band from either the sensitive or resistant primer set, but not both) using 18 cycles on the first (A & NE1) PCR reaction. The remaining 3 samples gave clear results when the cycle number for the A & NE1 PCR was increased from 18 to 30 cycles (data not shown), presumably due to a low amount of viral DNA in those samples. We are currently evaluating additional modifications to the protocol to increase sensitivity on those samples were detection is problematic.

PCR Reactions - 215 Mutation Detection

Use A(35) and NE1(35) primers for the first set of cycles and the B and either 3M or 3W primers for the second set of cycles to det ect mutant (resistant) or wild type (sensitive), respectively. These primers are identical to those described by Larder and Boucher (B. Larder, personal communication[38]). A third primer, Control (C) has been designed that lacks the terminal discriminating nucleotides found in the 3W or 3M primers. This primer will amplify either wild type (AZT sensitive) or mutant (AZT resistant) equally, and serves as an amplification control for the

second (nested) PCR step. The primer sequences are given below. The NE1(35), 3M, 3W, and C primers are 5' biotinylated.

A(35)	TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT
NE1(35)	CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT
B	GGATGGAAAGGATCACC
3M	ATGTTTTTTGTCTGGTGTGAA
3W	ATGTTTTTTGTCTGGTGTGGT
C	ATGTTTTTTGTCTGGTGTG

The PCR cycle part of the assay is identical whether the source material is plasma or tissue culture supernatants (viral RNA), or patient PBMCs or co-culture cells (proviral DNA). Sample preparation steps are given for each substrate. The preparation of viral RNA is identical to that used for the Quantitative RNA protocol described previously.

Sample Preparation: PBMCs or Co-cultured Cells

1. Thaw frozen cells at 37°C and transfer to a sterile 15 ml polypropylene centrifuge tube.

2. Wash once with 10 ml PBS (2000 rpm 15 min.). Decant supernatant after wash and discard.

3. Add lysis buffer (10 mM Tris 8.3, 50mM KCl, 2.5mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, proteinase K at 120 µg/ml) and resuspend pellet well for a cell concentration of 6 X 10^6 cells/ml. Be sure to lyse a negative control with the cell samples. Vortex briefly.

4. Incubate at 55°C - 60°C for 1 hr. Vortex before transfer in next step.

5. Transfer to 1.5 ml screw-cap microcentrifuge tube.

6. Heat-inactivate the proteinase K by incubating the tubes at 95°C for 15 min.

7. Transfer tubes to ice. Store lysates either at 4°C (no more than overnight) or at -20°C for longer periods.

Sample Preparation: Viral RNA from Plasma

Preparation of Plasma from Whole Blood

1. Centrifuge the whole blood at approximately 200 X g (1400 rpm) in a

tabletop centrifuge at 4°C.

2. Remove the supernatant, taking care not to disturb the cell layer.

3. Centrifuge the supernatant from Step 2 at approximately 1000 X g (4000 rpm) in a tabletop centrifuge at 4°C. The purpose of this step is clarification, to remove residual cells.

4. If the plasma is to be extracted immediately, maintain on ice. If it is to be stored, aliquot into 1.0 ml cryo-vials, and store at -70°C.

Pelleting of Virus

1. Add approximately 2 ml PBS/BSA (PBS containing 5 µg/ml BSA) to a Quick-Seal polyallomer ultracentrifuge tube. Alternately, a microfuge can be used to collect virus by pelleting. In this case, 2 volumes of PBS/BSA are added to 500 µl of plasma in a 2.0 ml screw-capped microfuge tube. Be sure to record the exact volume of plasma used at this stage if other than 500 µl.

2. Pellet the virus by ultracentrifugation in the 50.3Ti rotor at 40,000 rpm at 4°C for 1h, or similar run conditions in a similar rotor. If using a microfuge, spin at top speed (>10,000 xG) for 30 minutes at 4°C.

RNA Extraction

1. Remove the supernatant from the ultracentrifuge tube, transferring to a fresh, sterile microcentrifuge tube.

2. Add 800 µl Tri-Reagent (guanidinium/phenol). Vortex 15 s.

3. Allow to sit at least 5 min. at room temperature.

4. Add 160 µl $CHCl_3$ to each tube. Vortex 15 s.

5. Allow to sit at least 3 min. at room temperature.

6. Centrifuge at maximum speed (approximately 10,000 X g) in a microcentrifuge at 4°C for 3-5 min.

7. Remove the aqueous (upper, colorless) phase to a fresh tube.

8. Add 400 µl isopropanol (IPA, 2-propanol) and 5 µl of 2 µg/µl glycogen to each tube. Mix well by vortexing.

9. Maintain at -20°C overnight. Shorter times (between 1 hour and overnight) can be used will good, but less than optimal recovery of the RNA.

10. Centrifuge at maximum speed in a microfuge at 4°C for 30 min.

11. Decant the supernatant.

12. Wash the pellet with 1 ml of ice-cold 75% ethanol. Vortex briefly.

13. Centrifuge at maximum speed (approximately 10,000 X g) in a microfuge at 4°C for 30 min.

14. Decant the supernatant.

15. Air dry the pellet. Do not use a Speed-Vac.

16. Add Virus Lysis Buffer A (2.5% NP-40, 0.2 mg/ml tRNA, 1 U/µl RNasin, 2 mM DTT) for 10 µl per 0.1 ml original specimen volume. At least 50 µl of reagent should be used, regardless of specimen volume. Vortex.

17. Maintain at 42°C approximately 15 min. to fully dissolve the RNA, after which the extract should be stored at -70°C. The RNA should not be heated above 60°C until after reverse transcription, as RNasin in the Virus Lysis Buffer will be inactivated.

215 PCR Reaction Setup

The first set of PCR cycles uses A(35) & NE1(35) primers (NE1(35) primer is biotinylated) to produce a 805 bp fragment encompassing virtually all currently known drug resistance associated mutations in the HIV RT gene (amino acids 5-254 of RT).

PCR master mix:

37.1 µl H2O
22.4 µl dNTP (280 µM dNTP) (use dUTP only in the second PCR)
10.0 µl 10X 215 PCR buffer
10.0 µl Primers [A(35) & NE1(35); 0.2µM each.]

0.5 µl Taq polymerase (Promega 5 units/µl)

80.0 µl
70.0 µl oil overlay (may be omitted for PE 9600 cycler)
20.0 µl sample lysate

NOTE: In contrast to the PCR protocol in use for HIV detection in our laboratory, dUTP and UNG are not used in the first PCR of this nested set. The use of dUTP significantly reduces the discriminating power of the 3W and 3M primers used in the second PCR reaction.

The 10X 215 PCR buffer contains:

50 mM Tris 8.3
25 mM KCl
2.0 mM $MgCl_2$
100 µg/ml BSA

1. In the positive control lab, add positive controls (10^4 sensitive and/or resistant cells) from freshly thawed stock dilutions to the appropriate PCR tubes.

2. Immediately carry the reactions to the cycler.

PCR Cycling Conditions for Perkin Elmer 9600 Cycler

First (Outer) PCR Reaction Cycling Conditions

1. 94°C 1' 15"
2. 94°C 30"
3. 55°C 30"
4. 72°C 2'
5. Repeat steps 2-4 for 18-30 cycles.

The precise number of cycles depends somewhat upon the expected number of infected cells. When testing co-cultured cells, 18 cycles is usually sufficient, due to the large number of infected cells in the population, whereas primary patient cells often require 30 cycles, while up to 40 cycles may be used to generate more product if needed for cloning and sequencing. Since there is no UNG in these reactions, they may be maintained at 25°C after last cycle until products are ready to be carried into second PCR. Yield of PCR products may be monitored by running a 10 µl aliquot of the first PCR reaction on an agarose gel. It should be noted that in cases of low

numbers of virus, such as seen in patient PBMCs, no band may be visible after the first PCR. This is not necessarily an indication that the PCR failed however.

Reaction Setup for Second (Nested) PCR

The second (nested) PCR reaction utilizes the B and either 3W or 3M primers to discriminate between the wild type (AZT sensitive) or mutant (AZT resistant) genotype at amino acid 215 of the HIV-1 RT gene. The 3W primer recognizes either the Phe or Tyr mutant at amino acid 215 approximately equally. The C primer is identical in sequence to the 3W and 3M primers, save that it lacks the terminal 2 nucleotides that serve as sequence discriminators. This primer will amplify the resistant or sensitive genotype equally and serves as an amplification control, to evaluate PCR product yield and potential inhibition in the samples.

1. Remove 10 µl of the reaction and dilute 1:20 to 1:500 in lysis buffer.

NOTE: The exact dilution can be varied to ensure clean discrimination between 3M and 3W primer products. In general, there is less than a 100X difference in the product yield between completely homologous primer/template combinations (sensitive virus DNA with 3W primer for example) and mis-matched primer/template combinations (sensitive virus DNA with 3M primer) (Frank White, unpublished observations). Because of this, if the quantity of product transferred into the second PCR (B & 3M/3W) is too high, cross-reactive bands appear in both sensitive and resistant reaction lanes. In this case, it is necessary to either dilute the products of the first PCR reaction further and repeat the second PCR, or repeat the first PCR with reduced cycle numbers. This latter approach generally gives slightly cleaner results, but is also more time and labor intensive. The second PCR is set up exactly as the first, with the addition of 10 µl of first reaction product and 90 µl of master mix containing the B and 3M, 3W, or C primers. Note that dUTP can be substituted for dTTP in this reaction without affecting the specificity or sensitivity of the PCR. Uracil-N-glycosylase (UNG) may then be added to facilitate contamination control, as is the standard procedure for HIV detection PCR reactions.

PCR Master mix for second PCR (B & 3M/3W primers)

46.3 µl H_2O
20.0 µl dNTPs (250 µM dNTP. May substitute dUTP for dTTP)
10.0 µl 10X 215 PCR buffer
10.0 µl primers (B & 3W, 3M, or Control; 0.46µM each final)
3.0 µl $MgCl_2$ (25 mM stock, 2.25 mM final concentration)
0.2 µl UNG (0.2 units; Epicentre Technologies)

0.5 µl Taq polymerase (Promega, 5 units/µl)

90.0 µl total
70.0 µl oil overlay (may be omitted for PE 9600 Cycler)
10.0 µl diluted products from first PCR.

NOTE: There are slight changes in the concentrations of some of the reaction components (2.25 mM $MgCl_2$, 0.46 µM primers) between the first and second PCR reactions. These conditions have been optimized to increase product yield of the second PCR reaction.

Second (Nested) PCR Reaction Cycling Conditions

1. 94°C 5'
2. 94°C 1'
3. 48°C 30"
4. 72°C 30"
5. Repeat steps 2-4 for 30-40 cycles. Soak at 72°C after last cycle until products are either stored (-20°C) or analyzed.

PCR Product Analysis

After the second PCR, 10 µl of the products are analyzed on a 3% agarose gel run in 1X TBE containing EtBr. Results are seen as bands in the lanes corresponding to reactions containing either the B/3W (sensitive) or B/3M (resistant) primers. An example of this gel was previously shown in Figure 5. A mixture of sensitive and resistant virus would be seen as bands in both lanes. However, this may also indicate that the second PCR reaction was overloaded with product from the first PCR. To eliminate this problem, the number of cycles for the first PCR can be reduced (from 30 to 15-22) and/or the dilution of products from the first PCR increased from 1:20 to 1:100 or even 1:500 if needed to produce a single band from one or the other PCR reaction.

Application of the 215 PCR Assay to Clinical Samples

To date, over 300 patient PBMC samples and nearly 100 patient plasma (RNA) samples have been analyzed using the improved 215 PCR assay as developed by SRA Technologies, with additional samples currently being tested. The data from these samples has been presented to Dr. Douglas Mayers at WRAIR Division of Retrovirology and will not be repeated here. In summary, the protocol has been useful for following the progression of resistance in patients undergoing long-term AZT therapy. The results from many of the patients clearly indicates a progression

from AZT sensitive genotype at amino acid 215 to a mixed population (partially sensitive and partially resistant), and to a primarily resistant genotype at that position as therapy continued.

Ongoing ARMS Assay Development

One major limitation of the currently used agarose gel based assay is that it does not allow accurate evaluation of patient samples that contain a mixture of resistant and sensitive virus. Theoretically, the presence of a mixture of resistant and sensitive virus in a patient sample would produce PCR products from both the resistant and sensitive primer sets. These would be seen as bands appearing in both sets of lanes on a gel. Due to incomplete inhibition of primer extension from mismatched primers (such as the 3W primer hybridized to a resistant virus) however, it is possible to produce diagnostic bands of the gel from both the sensitive and resistant PCR reaction from samples that contain only one species of virus by simply overloading the second PCR reaction with product from the first (A & NE1 primer set) PCR reaction.

Traditionally, in order to resolve the problem of interpretation of bands appearing in both the sensitive and resistant reactions, the PCR products from the second reaction were serially diluted and re-analyzed on a gel. At the point that the PCR products from one primer set visually disappeared while the other remained, however faint, on the gel, the sample was considered to be composed entirely of that species. Only when both the resistant and sensitive bands disappeared at the same dilution was the sample considered to contain a true mixture. Obviously this evaluation is very subjective and cannot accurately assess any mixtures other than in 1:1 ratio, rendering it ineffective for many patient samples.

Currently we are pursuing additional modifications to the 215 protocol with the intention of increasing the sensitivity of the assay when analyzing plasma RNA samples, and to improve the quantitative ability of the assay. Toward these ends, we are experimenting with using more highly purified PCR primers, in the theory that if the PCR primer species is more uniform, with fewer oligo failure sequences in the preparation, that mis-extension from a mismatched primer will be reduced. Additional modifications to primer ratios and concentrations are being evaulated to improve the quantitative aspects of the assay, and equalize the amount of PCR product produced from each primer set. Our previous experience indicates that the resistant primer matched precisely with a resistant template produces about 2-3 times more product than a perfectly matched sensitive primer with a sensitive template. In order to accurately quantitate the proportion of the population that possesses each genotype, more equivalent yields are required from each control.

Although AZT is currently the most widely prescribed anti-HIV drug, other drugs and combination therapies are in increasing use as therapeutics. Each of these drugs, or combinations of drugs has its' own particular genotypic change associated

with the acquisition of resistance to the drug. It is anticipated that the 215 protocol can be easily modified to analyze additional mutations such as those at amino acid 74 associated with ddI and ddC, 181 associated with Nevarapine, and 236 associated with the Upjohn U90 family of compounds.

Diagnostic DNA Sequencing

Despite the effectiveness of the 215 PCR protocol described in the previous section, it is limited in that it can only be used where the site of mutation is known, and then only when the surrounding sequence is conserved sufficiently to ensure efficient primer hybridization. For new drugs, where the site of the resistance-conferring mutation is not well characterized, or for mutations occurring in hypervariable regions, diagnostic sequencing is the only method that can provide useful genotypic information.

While the basic technology of DNA sequencing has been around for more than 15 years, several relatively recent developments have lead to more extensive application of this technology beyond the research laboratory. PCR-based cycle sequencing using a variation of the Sanger protocol[39] allows direct determination of the nucleotide sequence from virus found in small volumes of patient blood, without the need for prior cloning of the sample[40]. The emergence of automated DNA sequencers[41] have facilitated the high throughputs required to support clinical trial using real-time monitoring of sequence changes that might be associated with the emergence of drug resistance during anti-viral treatments[42]. Combined, these techniques have moved DNA sequencing from its place in a limited setting research facility to a more broad based setting including clinical usefulness.

There can be a significant difference in approach between DNA sequencing used for diagnostic purposes, and when it is used to accumulate megabases of contiguous sequence information, such as for the Human Genome project. For example, in order to provide real-time patient monitoring during clinical trials, rapid turnaround time is essential. This in itself may preclude the traditional approach of cloning each template of interest prior to sequencing. Additionally, for diagnostic sequencing, often only small regions need be monitored for mutations, typically less than 100 base pairs. This reduces the need for long gel runs to build long contigs, and increases the need for protocols and sequencing hardware that can produce multiple runs a day for higher throughput. The ability to compare 5-10 sequences taken over time from a given patient during therapy is also very helpful to follow the accumulations of the mutations.

While there are many variations on sequencing protocols, the use of PCR to generate the DNA templates for sequencing has several advantages. Since HIV infects only a small percentage of the susceptible cell population, the viral DNA would be present at low copy number in a high background of host DNA.

Furthermore, there is some evidence that multiple strains of HIV, possessing characteristic nucleotide sequences at particular positions may develop or co-exist within an individual[43]. With its exquisite sensitivity, PCR can amplify the DNA from a single target cell to levels adequate for detection by one of several methods, including DNA sequencing. This makes it ideal for monitoring a patients progress using small volumes of blood taken at regular intervals. When more DNA is available, such as from cloned plasmid samples, other protocols, including the Sequenase protocol from USB can be used to generate long reads with high clarity[44]. One protocol currently in use in our laboratory for the purification of PCR products prior to sequencing is given below. Two alternate methods of DNA sequencing are also given, the choice of protocol being determined by the type and quantity of DNA to be sequenced as well the amount of sequence information required to characterize the sample.

Quality Control for DNA Sequencing

When using PCR based sequencing techniques, several potential problems may arise. Several studies have examined the error rate of *Taq* DNA polymerase when used in a PCR assay[45-47]. The use of a recently introduced thermostable DNA polymerase (*Pfu* DNA polymerase, Stratagene Inc.), with a 3'-exonuclease proofreading activity, should significantly reduce the chance of PCR induced errors in sequence determination[48]. In collaboration with Stratagene, work is ongoing at SRA Technologies to evaluate *Pfu* polymerase for use in some of our PCR protocols, as well as for use in DNA sequencing.

While this potential for error could conceivably be a problem during the initial PCR reactions, it will not be a factor during the actual sequencing reactions since the products seen on a gel image after sequencing actually represent a large population of molecules at each nucleotide position on the gel. A single, random, enzyme-induced sequence alteration would be lost in the background of the assay. However, if required, multiple aliquots and/or both strands of the template should be sequenced to ensure that the sequence represented is accurate. For PCR-based sequencing of rare templates, similar procedures (i.e., separation of laboratory work areas and equipment, use of UNG in the PCR reactions where possible) should be followed as are used for diagnostic PCR to ensure that sequence information is indeed derived from the virus isolate of interest.

PCR Product Purification using Dynabeads™

If PCR reactions are run using a biotinylated primer, as is the routine at SRA Technologies, the products may be easily purified using avidin-conjugated solid supports[49-51]. Dynal beads (avidin-conjugated supraparamagnetic beads) are currently the method of choice in our laboratory for PCR product purification. A non-biotinylated primer is used for the sequencing reactions. The protocols given

in the following sections utilize ^{33}P in the sequencing reactions, but ^{35}S has also been used with good success, and may be substituted with only slight changes to the protocols. Although we anticipate purchasing a fully automated fluorescent sequencer within the next few months, we still foresee an important role for isotopic manual sequencing in our laboratory.

Preparing the Dynabeads

1. Place 30 µl Dynabeads (Dynabeads M-280 Streptavidin, 10 mg/ml) in a 1.5 ml centrifuge tube. Place tube in magnetic separator (Dynal MPC-1), wait for beads to adhere to side of tube (30 seconds), and remove supernatant with a micropipette.

2. Resuspend beads in 30 µl B&W buffer (10 mM Tris-HCL (pH 7.5; 1 mM EDTA; 2.0 M NaCl) Place tube in the magnetic separator (30 seconds), remove supernatant and discard it.

3. Resuspend beads in 60 µl of B&W buffer.

Immobilization of PCR Products

1. Add total PCR product (approximately 100 µl) to the 60 µl of prewashed Dynabeads. (Be sure to avoid any transfer of mineral oil from the PCR reaction if it was used.)

2. Incubate at room temperature for 15 minutes. Dynabeads should be resuspended occasionally during incubation by gently tapping the tube.

Denaturing the Double-Stranded PCR Products

1. Place the centrifuge tube in the magnetic separator for 30 seconds and remove supernatant.

2. Wash Dynabeads with 40 µl B&W buffer.

   NOTE: Dynabeads can now be stored at 4°C for several weeks.

3. Remove supernatant and resuspend Dynabeads in 10 µl of 0.1 M NaOH.

4. Incubate at room temperature for 10 minutes.

Separating the Strands

1. Collect Dynabeads in magnetic separator and transfer the NaOH supernatant (containing the non-biotinylated strand) to a clean tube. Add 350 µl TE buffer and transfer the unbound strand to a Microcon (Amicon) 30 or 100 microconcentration unit (similar units may be substituted for the Microcon).

2. Wash Dynabeads once with 50 µl 0.1 M NaOH. Remove supernatant and transfer this to the Microcon unit.

3. Wash Dynabeads once with 40 µl B&W buffer. Remove supernatant and discard.

4. Wash Dynabeads 3 times, each time using 50 µl TE buffer, removing supernatant between each wash and discarding it.

5. Resuspend Dynabeads in 6 µl sterile ultra pure water for sequencing.

Purifying Non-Biotinylated Strand

1. Microcentrifuge Microcon 100 at 3,000 RPM for 9 minutes.

2. Wash Microcon 100 three more times, using 350 µl ultra pure water each time, repeating the spin after each wash.

3. Turn retentate cup upside down and place into a clean catch tube. Microcentrifuge 4 minutes at 1,000 RPM's to recover template.

4. Recovered template is lyophilized and resuspended in a final volume of 5 µl sterile ultra pure H_2O.

Purified PCR products may be sequenced using either USB Sequenase or by a cycle sequencing protocol. An example of each is given below.

Sequencing Protocol Using USB Sequenase™ Version 2.0 T7 DNA Polymerase

The USB Sequenase kit is the standard by which all other DNA sequencing protocols are measured due to its consistent band intensity and processivity. When coupled with multiple loadings and an "extended resolution" gel matrix, such as Long Ranger™ acrylamide, over 500 bases of sequence can be read from a single gel. Similar results are seen using automated fluorescent DNA sequencing systems[52]. The current protocol in use in our laboratory is given below. All reagents are used as provided in the Sequenase kit, unless otherwise noted. All sequencing primers are non-biotinylated. Although the protocols given here are for standard isotopic methods, the chemistries are easily adapted to fluorescent and automated sequencing procedures as well, primarily by substituting the radioactive label with a fluorescent one.

Annealing Template and Primer

1. For each template combine in a 1.5 ml microcentrifuge tube:

   Dynabeads-Template complex in 6 ul water
   1.0-2.0 pmole Primer
   2 µl Reaction Buffer
   Ultra pure water to 10 µl

2. Warm the capped tube in 65°C heat block for 2 minutes.

3. Take the heated block out of the heating unit and place on counter to allow for slow cooling over a period of 30 minutes. Do not let temperature fall below 30°C.

4. After 30 minutes, place tube on ice.

5. Dilute Sequenase T7 DNA polymerase enzyme 1:8 in dilution buffer. Keep on ice until ready for use.

Labeling Reaction

1. To the annealing template - primer reaction add the following:

   1.0 µl DTT
   2.0 µl labeling nucleotide mix diluted 1:5
   0.5 µl ^{33}P (~5 µCi)

2.0 µl of Sequenase Enzyme diluted 1:8 (3 units)

2. Total volume should be approximately 15 µl. Mix well by briefly (3 seconds) centrifuging and incubate for 2-5 minutes at room temperature.

Termination Reactions

1. Label 4 tubes "A","G","C" and "T". Add 2.5 µl of the appropriate dideoxy termination mixture to each labeled tube.

2. Pre-warm these tubes at 42°C for 1 minute in heat block before use. (Avoid temperatures below 37°C)

3. Transfer 3.5 µl of the Labeling Reaction to each of the pre-warmed "A","G","C" and "T" tubes. Mix well by pipetting up and down several times.

4. Incubate tubes for 2-5 minutes at 42°C, and then add 4 µl stop solution to each tube. Mix well and immediately place on ice.

5. Before loading the gel, heat samples to 75-85°C for 5 minutes in heat block. Quickly place on ice. Place centrifuge tube in magnetic separator, pull off 4 µl supernatant and load on gel.

Sequenase Reaction Solutions

Reaction Buffer
- 200 mM Tris-HCL (pH 7.5)
- 100 mM MgCl
- 250 mM NaCl

Dithiothreitol (DTT)
- 0.1 M

Labeling Nucleotide Mixture
- 1.5 µM dGTP
- 1.5 µM dCTP
- 1.5 µM dTTP

Deoxy/Dideoxy Sequencing Mixes (µM)

Mix	A Mix	C Mix	G Mix	T Mix
ddATP	8	-	-	-
ddCTP	-	8	-	-
ddGTP	-	-	8	-
ddTTP	-	-	-	8
dATP	80	80	80	80
dCTP	80	80	80	80
dGTP	80	80	80	80
dTTP	80	80	80	80

Stop Solution/Loading Buffer

95% Formamide
20 mM EDTA
0.05% bromophenol blue
0.05% xylene cyanol FF

Enzyme Dilution Buffer

10 mM Tris-HCL (pH 7.5)
5 mM DTT
0.5 mg/ml

Cycle Sequencing Using NEB CircumVent™ Polymerase

Cycle sequencing has the potential advantage of requiring significantly lower amounts of template than do conventional sequencing methodologies[53-55]. When attempting to obtain viral DNA sequence directly from patient samples this advantage could prove critical. In addition, by examining patient cells directly, the chance for culture passage induced mutation is eliminated. We are actively pursuing this approach and are currently evaluating several commercially available cycle sequencing kits. To date, the NEB CircumVent™ kit has produced the best results in our laboratory. The current protocol is given in this section. Unless otherwise noted, all reagents are from the NEB CircumVent™ cycle sequencing kit. All sequencing primers are non-biotinylated. We have also gotten excellent results using several other commercially available kits, including the *Pfu* exo- cycle sequencing kit from Stratagene and are continuing evaluation of new cycle sequencing kits as they become available. This protocol is also easily adapted to fluorescent systems by the substitution of the radioactive label by a fluorescently labeled primer. Direct incorporation of fluorescent labels (dye-terminator chemistry) is also supported.

Preparation of Deoxy/Dideoxy Reaction Tubes

1. Label four 0.5 ml centrifuge tubes A, G, C and T.

2. Add 3 µl of deoxy/dideoxy sequencing A mix to the bottom of the A tube, and 3 µl of G, C and T mixes to the bottom of the G, C and T tubes, respectively.

Combining Template and Primer

1. In a 0.5 ml centrifuge tube, combine the following:

   0.01 pmol double-stranded template DNA
   1.2 pmol primer
   1.5 µl 10x CircumVent sequencing buffer
   1.0 µl 30x Triton X-100 solution
   ultra pure water to total volume of 12.0 µl

2. Mix by pipetting. Briefly (3 seconds) centrifuge.

Labeling and Termination Reactions

1. To the tube containing template, primer and buffer, add 2 µl of ^{33}P dATP.

2. Add 1 µl of Vent (exo-) DNA polymerase and mix. Briefly (3 seconds) centrifuge.

3. Immediately distribute 3.2 µl of this reaction to the tube containing the A termination mix (tube labeled A) and mix by pipetting. Repeat this for G, C and T tubes. Be sure to change pipette tips each time.

4. Overlay each termination reaction with 10 µl mineral oil. Briefly centrifuge (5 seconds).

5. Place tubes in PE 9600 thermal cycler and cycle as follows.

95 °C	20 seconds	
55 °C	20 seconds	20 cycles
72 °C	20 seconds	

6. When cycling is complete, add 4 µl Stop Dye Solution to each reaction, beneath the mineral oil. Place all tubes on ice.

7. Store at -20°C. When ready to load gel, heat tubes at 75-80 °C for 5 minutes and place on ice.

8. Load 2-4 µl of each reaction into a well on a thin (0.2-0.4 mm) denaturing polyacrylamide-urea gel and electrophorese for 2 to 8 hours at 45-55°C. SRA Technologies is currently using Bio-Rad Sequigen DNA sequencing electrophoresis equipment and Bio-Rad computer controlled high voltage power supplies. The Bio-Rad units use a special glass plate with the upper buffer chamber running the length of the gel plate, to ensure even heating across the gel surface. Coupled with the computer controlled power supplies, extremely reproducible sequencing gels are possible, greatly facilitating comparison of identical samples between runs. If, instead of ^{33}P dATP, ^{35}S dATP is used, the urea must be removed from the gel and the gel must be dried before autoradiography. This is done by soaking the gel in 5-10% methanol/ 5-10% acetic acid for 15-20 mins. followed by vacuum drying the gel. This step may be omitted if Long Ranger™ acrylamide (AT Biochem) is used. Kodak X-AR5 or Amersham ß-Max Hyperfilm x-ray film is exposed to the dried gel for 16 to 48 hours without an intensifying screen.

Sequence Reaction Solutions

10X Sequencing Buffer
- 100 mM KCl
- 100 mM $(NH_4)_2SO_4$
- 200 mM Tris-HCL, pH 8.8
- 50 mM $MgSO_4$

Stop Solution/Loading Buffer
- Deionized Formamide containing:
- 0.3% Xylene Cyanol FF
- 0.3% Bromophenol Blue
- 0.37% EDTA (pH 7.0)

Deoxy/Dideoxy Sequencing Mixes (μM)

Mix	A Mix	C Mix	G Mix	T Mix
ddATP	900	-	-	-
ddCTP	-	480	-	-
ddGTP	-	-	400	-
ddTTP	-	-	-	720
dATP	30	30	30	30
dCTP	100	37	100	100
dGTP	100	100	37	100
dTTP	100	100	100	33

Sequence Data Analysis

After extensive hand-on evaluation of several automated and semi-automated DNA sequence analysis systems, SRA Technologies has recently purchased an automated DNA sequence analysis system from Millipore/BioImage Inc. This system currently consists of gel reading and band quantitation software running under Solaris 3.0 on a Sun SPARC 10 workstation with 32 megabytes of ram and 1.1 gigabytes of hard disk storage on-line, with CD-Rom, optical disk, and tape drive available for off-line storage. Although this system is not as popular as the ABI 373 system in the USA, it has an extensive user-base overseas with an excellent reliability record, and has some unique capabilities not available on the ABI system. Most important of these are the abilities to make use of manually-generated sequence information along side with the automated fluorescent data, and the computing power to support multiple users at one time, including sequence analysis and protocol development at the same time as the system is acquiring new sequence data. These capabilities far exceed those of the Macintosh-based ABI 373 system. Additionally, the flexibility of the software and sequencing system allows custom tailoring of the sequencing protocols permitting multiple sequencing runs using varying parameters per day, exceeding the throughput capacity of the ABI system.

Currently, DNA sequence information is acquired with a Howtek Scanmaster 3+ flat bed scanner used to digitalize the entire 35X43 cm film of the sequencing gel. It is anticipated that the BaseStation automated sequencer will be added to the system within the next few months. The BaseStation system utilizes a CCD (charge-coupled device) camera to provide real-time image information of the self-contained sequencing gel running standard sequencing chemistry and fluorescently labeled primers or terminators. Using input data from either digitized films or directly from the BaseStation, the analysis software on this system can automatically define the sequencing reaction lanes and perform automatic base calling using neural network algorithms for increased accuracy. These advanced algorithms can be "trained" to improve base-calling accuracy the more they are used. In addition, the

software allows quantitation of band intensity from the sequencing gel, facilitating determination of mixed population at a particular base location based in the sequencing gel information.

This system is currently connected to our LAN (described in the reports section) and can access other computers both in-house and through the Internet. The system can also interact with other molecular biology software packages, such as the DNAStar Lasergene system currently running on a Mac IIci with Genbank on CD-Rom. Custom filters have been provided to directly access the Mac format DNAStar files for use on the SUN system. These capabilities will provide easy access to the sequence data generated in our laboratories, and the information generated can easily be included in reports requested by NIAID.

Application of Diagnostic DNA Sequencing to Clinical Samples

Recently we have started to apply this technology to confirming the results of the 215 PCR assay for the presence of mutations conferring AZT resistance. It is anticipated that its usefulness will increase significantly over the next couple of years, especially to support clinical trials of combination or novel drug therapies, where the location and number of resistance-conferring mutations will be more variable than single drug therapy with AZT.

Sequencing Protocol Development

Our laboratory has extensive experience fine-tuning sequencing protocols to obtain the maximum sequence information from each experiment. In addition, an ongoing research and development effort ensures familiarity with new technologies as they develop. SRA Technologies maintains contacts with several companies providing both hardware and reagents for DNA sequencing and is engaged in beta-testing programs with several of those companies to stay on the leading edge of technology development.

A potential problem in obtaining quality sequence information is the occurrence of template secondary structure and its inhibition of polymerase processivity. If this occurs, alterations to the basic protocol can easily be introduced. These may include varying the temperature of the extension reaction and the polymerase concentration, addition of Single Strand Binding (SSB) protein to facilitate template unwinding, and the use of 7-deaza-GTP or dITP in the place of dGTP[56]. With an optimum extension temperature of approximately 72°C, one of the thermostable DNA polymerases (*Taq* or Vent exo-) can be used to obtain sequence from regions refractory to sequencing at lower temperatures due to extensive secondary structure. The substitution of HydroLink Long Ranger acrylamide instead of conventional acrylamide:bis-acrylamide mixtures may be suggested due to its significantly increased resolution, greater mechanical strength, and reduced requirements for urea removal prior to autoradiography during manual sequencing. When

converted to a fully automated fluorescent system the problems associated with radioactive sequencing would obviously be eliminated.

No significant disruption in our ability to generate quality DNA sequence information is anticipated during the switch-over to a florescent system. Millipore has been working extensively with New England Biolabs (producer of the Vent *exo-* cycle sequencing kit) to ensure smooth migration from radioactive to fluorescent reactions. In addition, SRA Technologies has been collaborating with Millipore and Stratagene for ongoing sequencing protocol development prior to installation of the Basestation in our laboratories to ensure a smooth transition to that system.

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2. Cellular Phenotype Working Group

The Cellular Phenotype Working Group has worked in three major areas during the first contract year. These included; 1) developing phenotypic assays to support evaluation of neutralizing antibodies in vaccinees and cross neutralization studies for eventual establishment of subgroup vaccines, 2) developing *in vitro* systems to evaluate antiviral genes for the treatment of HIV disease and 3) performing in vitro antiviral drug studies and other phenotypic analyses of patient isolates in support of clinical HIV chemotherapy. The progress made during the past year in each of these areas is reviewed below.

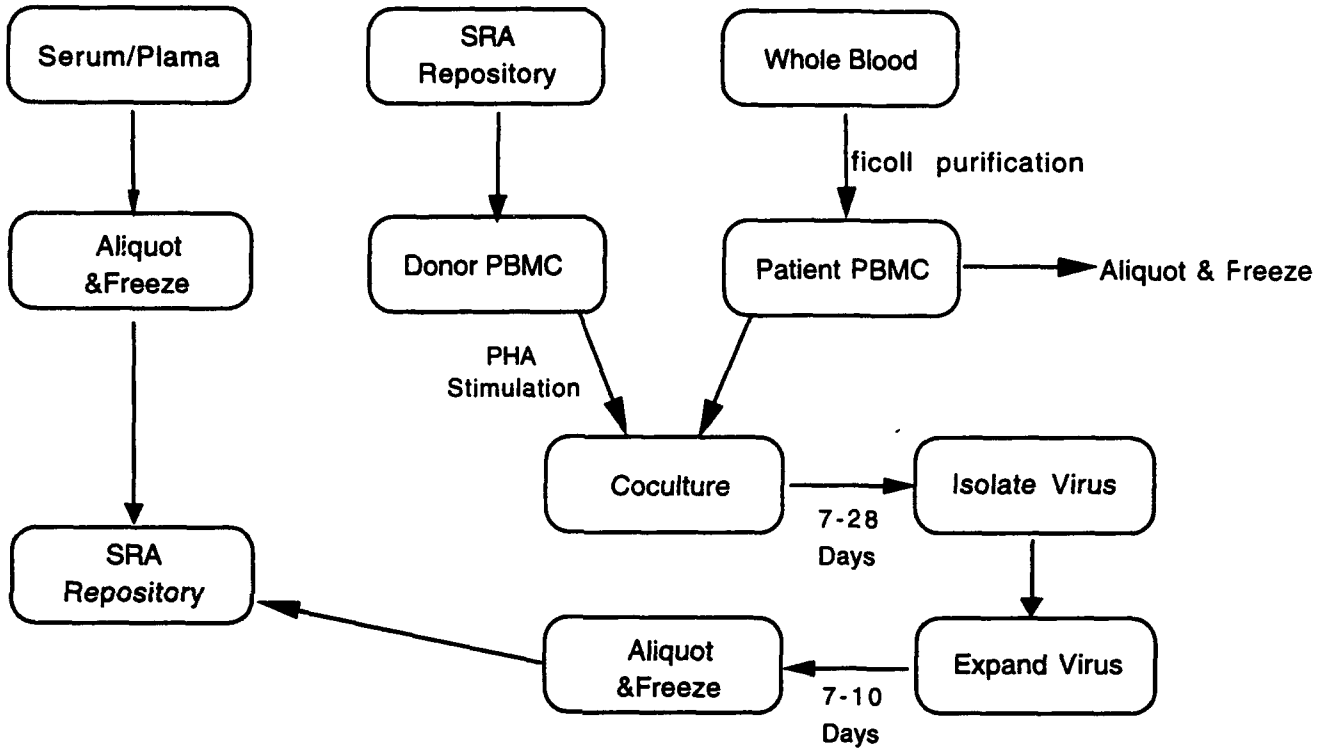
A. Development and Optimization of Virus Titration and Neutralization Assays

Virus neutralization assays may be employed to identify and differentiate virus, as well as to determine the host immune responsiveness to a specific viral infection and/or vaccination with various viral protein(s). Although identification of serum antibodies which inhibit viral infection *in vitro* may be a useful marker of protective immunity for some viruses *in vivo*[1], the significance of neutralizing antibodies in influencing clinical outcome in HIV infected individuals is not well understood[2,3,4,5,6,7,8]. Currently, there are a number of assays being used to evaluate the effect of antibody on HIV replication. Originally, most studies utilized immortalized cells (*e.g.*, H-9, MT-2, etc) that exhibited susceptibility to one or more laboratory strains of HIV (IIIb, RF, MN, etc). Susceptibility was usually evaluated by the production of viral markers and/or the induction of cytopathic effects (CPE).[9,10.11.12.13] However, most field isolates of HIV (*i.e.*, low passaged, patient isolates) infect immortalized cells with very low efficiency, thus these assays are of limited value in assessing the neutralizing antibody titers of a patients sera to clinical isolates. During the past contract year, efficient primary peripheral blood mononuclear cell (PBMC)-based assays were developed and optimized in collaboration with the Retrovirology Division of the Walter Reed Army Institute for Research (WRAIR).[14,15] Figures 6 to 8

Figure 6

Approach To Studies of Neutralizing Antibodies From HIV-Infected Patients or Vaccinees

1. Specimen Proccessing, Virus Isolation and Expansion



schematically summarize the approaches developed thru the contract for assessing neutralizing antibodies in patients or vaccinees utilizing materials supplied by WRAIR. Flowcharts describing the procedures employed have been provided elsewhere in this report as have illustrative data.

SRA's approach to virus neutralization and other methods of assessing humoral immune function assumes that we will be provided with the appropriate virus stocks and that virus titration will be required prior to the start of any testing. With that in mind we developed protocols for the selection of virus sensitive donor PBMCs and expansion and titration of early passaged isolates of HIV (Figs.6 and 7). Once virus stocks have been prepared and titered (in selected leukopack-derived PBMCs) we procceed with a neutralization screen (Fig. 7) of all sera and viruses in a checkerboard fashion or as directed by the WRAIR. Sera or plasma found active in this preliminary screening assay is further analyzed for specificity and determination of neutralization endpoints in a serum titration procedure (fig. 8).

Figure 7

Approach To Studies of Neutralizing Antibodies From HIV-Infected Patients or Vaccinees Continued:

2. Pre-Neutralization Virus Titration, Antibody Neutralization Screen

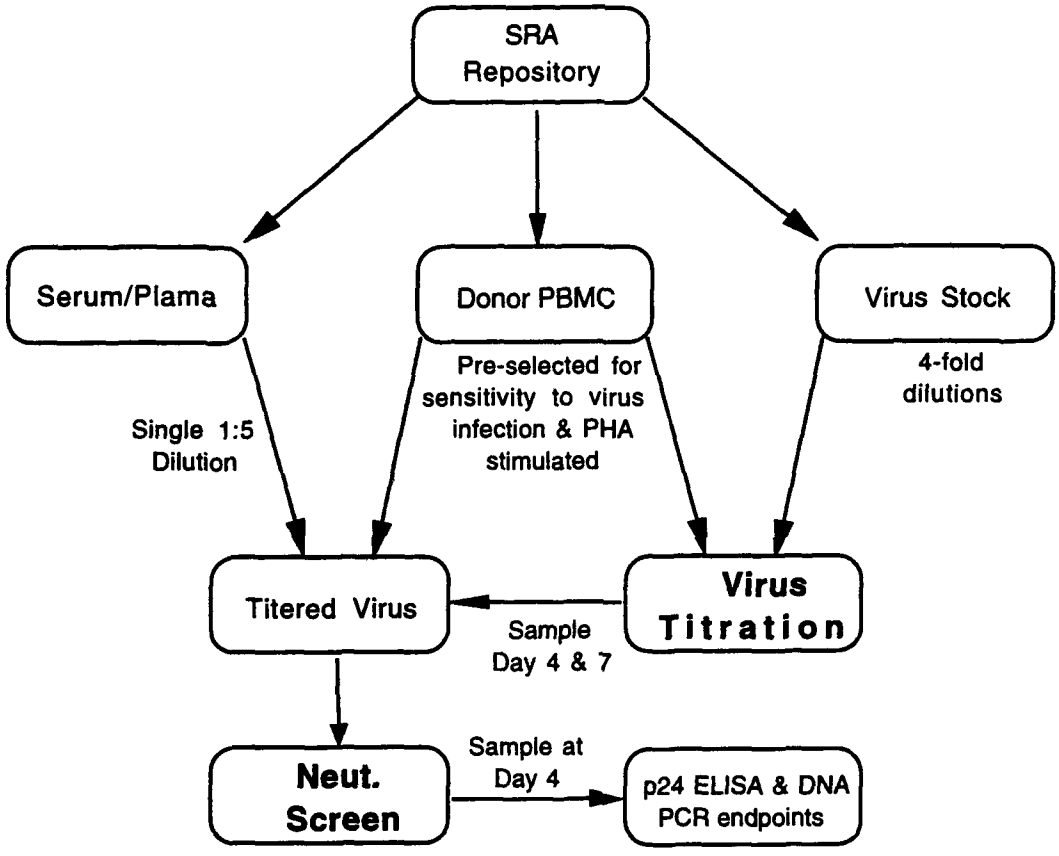
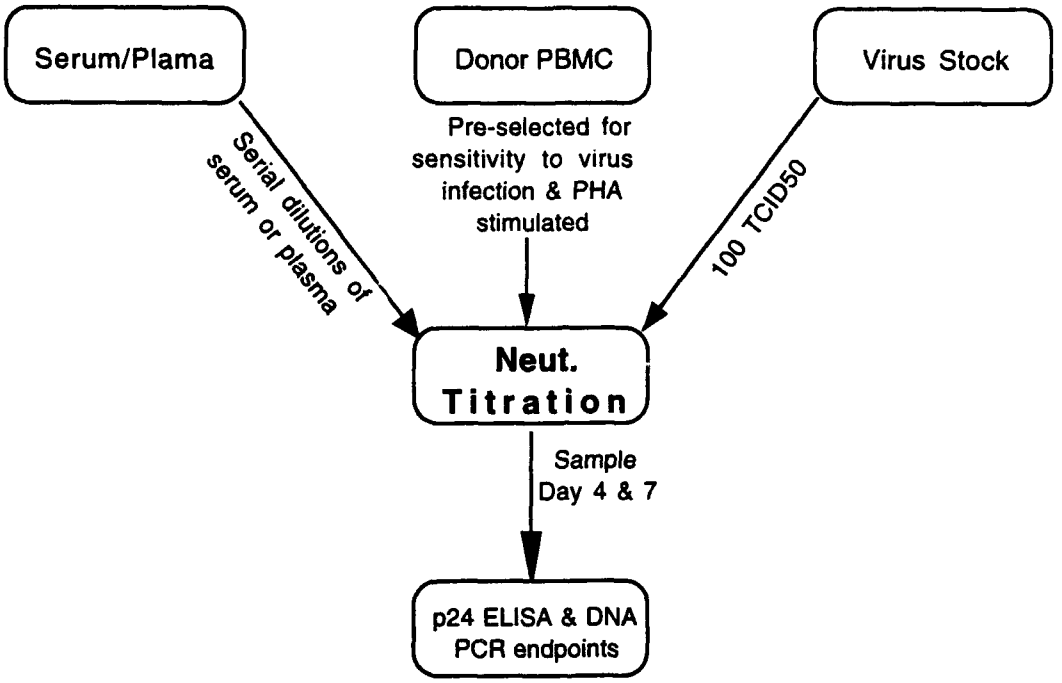


Figure 8

Approach To Studies of Neutralizing Antibodies From HIV-Infected Patients or Vaccinees Continued:

3. Serum/Plasma Titrations: Determination of Neutralization Endpoints and Antibody Specificity



A-1. Use of Pre-Neutralization Virus Titration to Assess PBMC Sensitivity to HIV Replication and Optimize Culture Systems

During the contract year we developed procedures for the evaluation of leukopack-derived donor PBMCs for sensitivity to HIV infection. This was important for two reasons. First, the asssays we were required to develop were short term systems usually of four days duration. This necessitated the production of measureable levels of HIV p24 (the endpoint for the majority of studies) in a short span of time. Secondly, many of the clinical isolates employed in this work were slow growers, producing only low levels of p24 over the required four day period of culture. Thus use of only the most virus sensitive PBMCs was required. Using a pre-neutralization virus titration procedure, developed during the past year, we analyzed a number of donor leukopack-derived PBMCs for sensitivity to virus infection by slow/low (from early stage patients) and rapid/high variants of HIV (from late stage

AIDS patients) on donor cells. The best donors, i.e. those that provide the highest titers in the shortest period of time are selected, in some cases pooled, and whenever possible, used throughout the entire process of virus titration, characterization and neutralization (or other immunoassay analyses) as required.

The data in Table 2 illustrate SRA's use of the aforementioned pre-neutralization titration protocol for evaluation of cyropreserved PBMCs obtained from eight normal donors. The cells were tested in a single experiment against four separate isolates of HIV-1. These isolates were donated by investigators at the Walter Reed Army Institute for Research (WRAIR) and the Henry M. Jackson Foundation (HMJF) who classified them as rapid/high (9881 and 873b) or slow/low (8871 and 4026).

Table 2

EFFECT OF DONOR PBMCs ON VIRUS TITER

PBMC DONOR	VIRUS ISOLATE			
	4026	8736	8871	9881
13108	1024*	2048	12944	131072
14486	NT	512	648	10369
14557	2048	1024	12944	>131072
14659	8192	3236	65536	>131072
16952	20738	1024	4096	>131072
17679	128	2048	676	32768
18072	809	648	12944	103552
18251	1024	512	5404	51776

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

The data suggest that a remarkable variability exists between donor PBMCs as regards their ability to support replication of these isolates. The reasons for this variability are not immediately evident.

As noted above, it is SRA's intention to utilize single leukopack donor-derived PBMCs for all phases of neutralization study including virus titration and neutralization. To ensure that sufficient numbers of PBMCs are available for *in vitro* studies we have evaluated a single donor's multiple bleeds, individually and as cell pools, for sensitivity to infection with rapid/hi (9881) and slow/low (4971) variants of HIV. The data generated in this study are summarized in Table 3 below. In one experiment leukopacks 20841, 21480 and 18251 were tested individually for sensitivity to virus in a pre-neutralization titration. Leukopacks 20841 and 21480 were clearly more sensitive to virus replication at days 4 and 7 when compared to 18251.

Table 3

EFFECT OF LEUKOPACK POOLS ON VIRUS TITERS

	Leukopack Pools				
		Exp 1		Exp 2	
Virus Isolate	20841	21480	18251	20481 21480	18251 20481 21480
9881					
Day 4	5,404*	6,562	2,048	26,249	4,096
Day 7	65,536	32,768	8,192	419,991	14,263
4971					
Day 4	NT	NT	NT	2,048	2,048
Day 7	NT	NT	NT	14,263	5,404

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

A second, independent, experiment was performed using pools of these 3 leukopacks to determine the influence of a poorly performing leukopack on a pool of the more sensitive cells. These data show that the addition of 18251, a less sensitive bleed, to a pool of 20481 and 21480 (better individual performers) significantly reduced the titers obtained at days 4 and 7 for the 9881 isolate. The mechanism responsible for this effect is not immediately clear, but initial observations suggest that adherent cell populations appearing on flask surfaces during PHA stimulation of 20481 and 21480 are markedly reduced during stimulation of 18251 and the 3 leukopack pool. These data again point to the importance of pre-screening all donor PBMCs prior to their use in neutralization assays.

Earlier studies in our laboratory have suggested that fewer numbers of cells per well at the start of culture result in better cell growth and higher titers of virus produced. The results in Table 4 summarize those initial studies and demonstrate that for rapid/hi isolates in particular

Table 4

Effect of Cell Density on Cell Proliferation and Virus $TCID_{50}$

Num of Cells/well		Fold Increase in Cell Numbers		$TCID_{50}$	
	Virus	Day 4	Day7	Day 4	Day 7
5×10^4					
	4971.5	8.8	12.8	891	1024
	9881			7131	10809
2×105	4971.5	2.9	2.9	675	445
	9881			2048	2048

* $TCID_{50}$ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Karber.

(9881 is but one example) fewer numbers of cells per well may result in significantly improved cell growth and higher early titers of virus. Indeed, prior to using any leukopack or pool of leukopacks these types of analyses will be performed to maximize use of available materials. During the past contract year the preneutralization procedure was used

to analyze some 25 individual and pooled leukopacks for virus sensetivity against slow/low and rapid/high isolates. Determinations of virus titers were made for no less than 18 international viral expansions and for at least 40 viruses in multiple leukopacks as a prelude to various experimental procedures. This work represented more than 8400 individual cultures of PBMCs.

A-2. Neutralization Screen and Serum Titration: Optimization and Illustrative Data

SRA developed and optimized two neutralization assays during the past contract year. The first is a rapid screening protocol capable of evaluating up to 17 test sera per 96 well microculture plate at a single dilution of 1:10 (final in the presence of virus). The second protocol, a serum or plasma titration procedure, is utilized to confirm neutralization, determine the precise titer of sera or plasma and define cross reactivities suggested by the neutralization screen. A simplified flow chart describing both procedures is presented below (figure 9). Examples of experimental data generated using these assays during FY93 are described in the following paragraphs.

During development of these assays we made a number of important observations regarding the neutralization of both laboratory and low passage, clinical isolates of HIV in PBMCs. Analysis of cell density (see table 4 above), virus x antibody incubation intervals and the interval of target cell infection in the presence of virus and antibody have assisted in efforts to optimize both the screening and serum titration procedures.

Although laboratory strains of HIV (IIIb for example) were readily neutralized in the early incarnations of the neutralization assay, we were unable to observe consistent neutralization of low passaged clincal isolates of HIV. We performed a number of experiments to determine the influence of incubation times on various neutralization steps and these are briefly summarized below. In the study illustrated in figure 10 the preliminary incubation of virus dilutions with antibody was maintained at 30 minutes; however, the subsequent infection period (antibody + virus + cells) was varied from one to sixteen hours prior to the washout of antibody and virus. Clearly a one hour incubation period is sufficient for neutralization of the

Figure 9

General Protocol for Neutralization

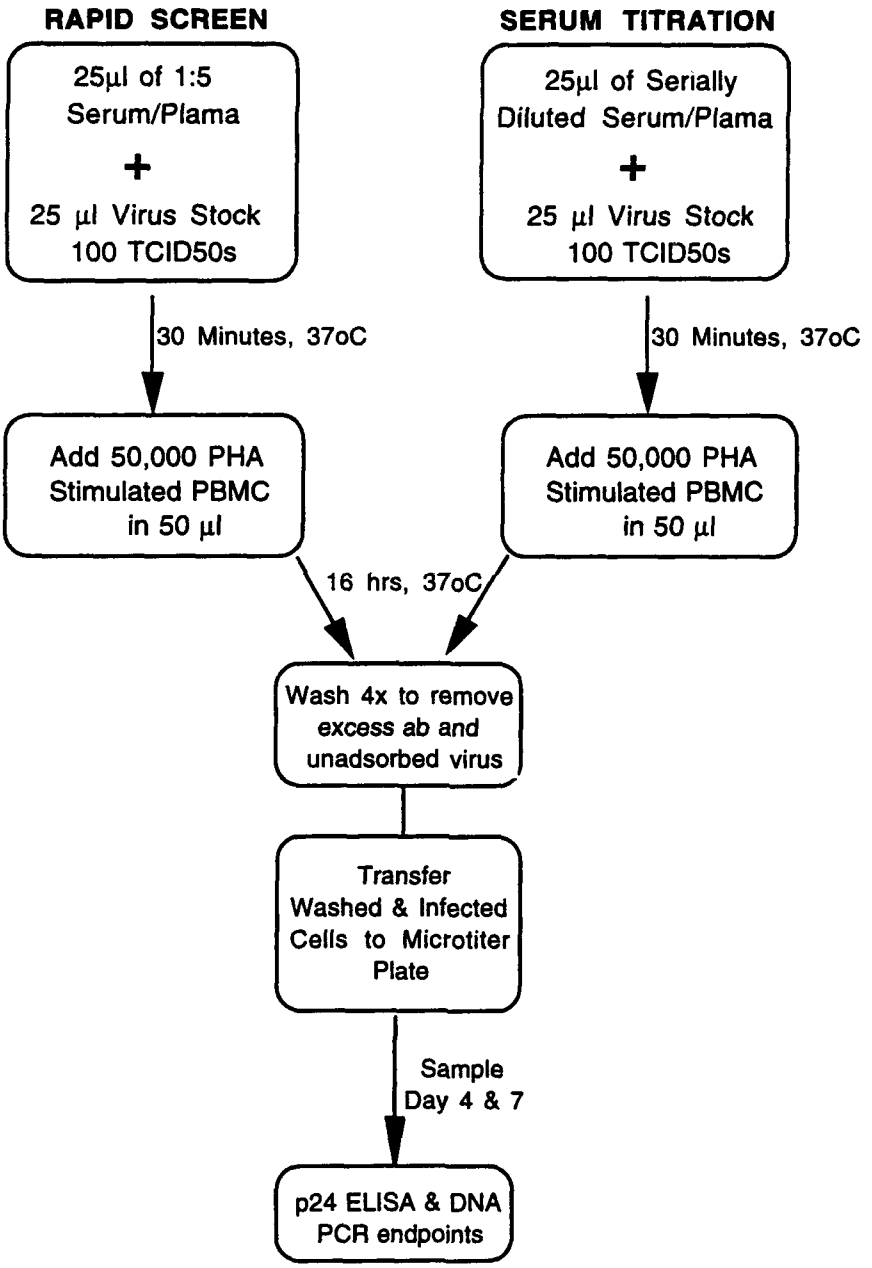
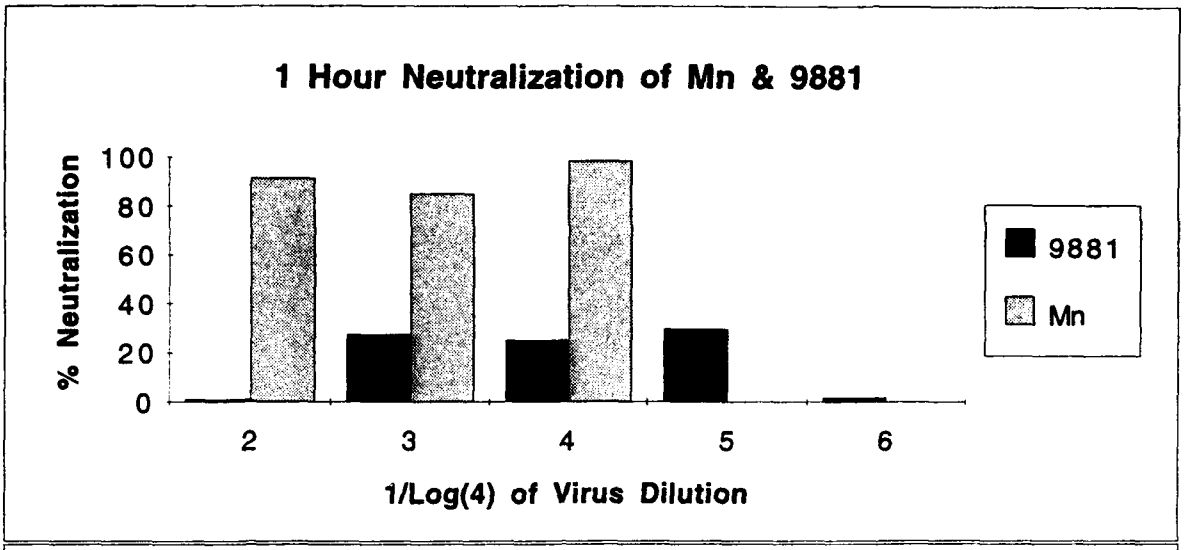
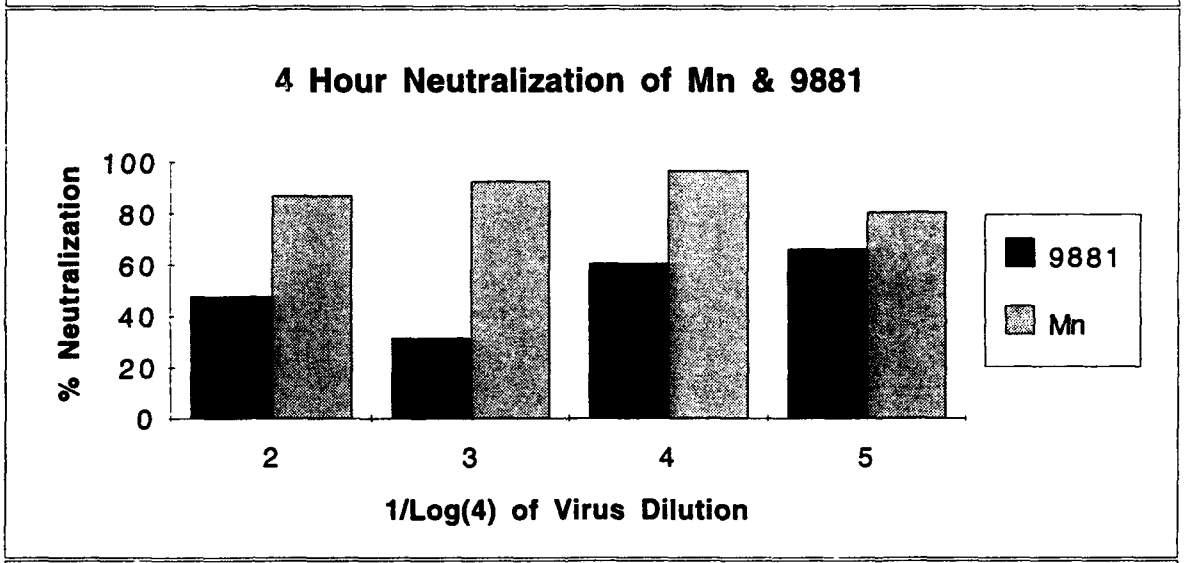


Figure 10


1 Hour Neutralization of Mn & 9881
% Neutralization
100
80
60
40
20
0
2
3
4
5
6
1/Log(4) of Virus Dilution
9881
Mn


4 Hour Neutralization of Mn & 9881
% Neutralization
100
80
60
40
20
0
2
3
4
5
1/Log(4) of Virus Dilution
9881
Mn

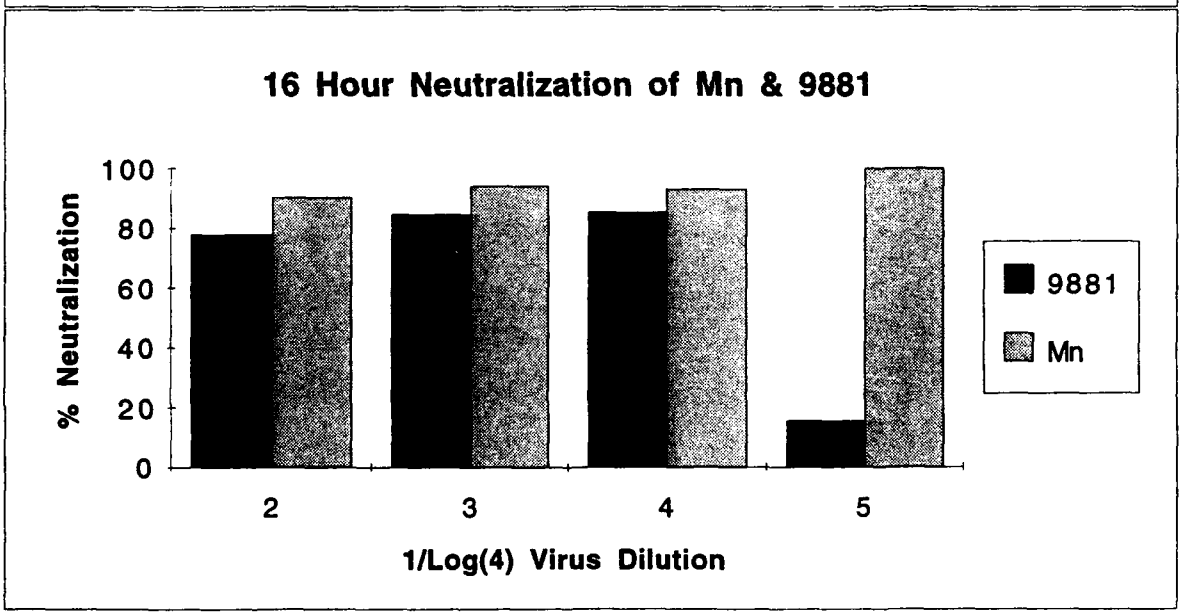

16 Hour Neutralization of Mn & 9881
% Neutralization
100
80
60
40
20
0
2
3
4
5
1/Log(4) Virus Dilution
9881
Mn

Figure 11

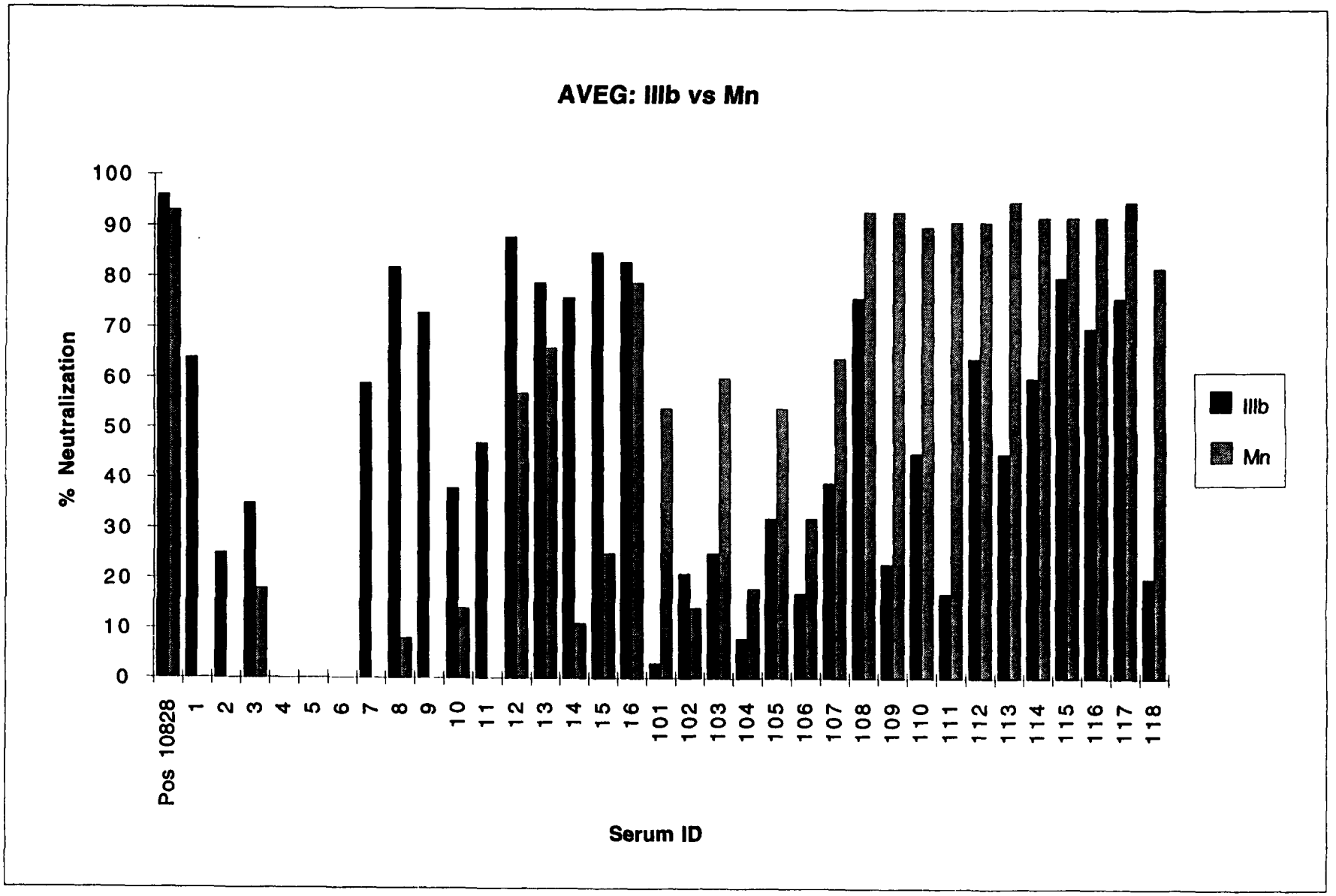
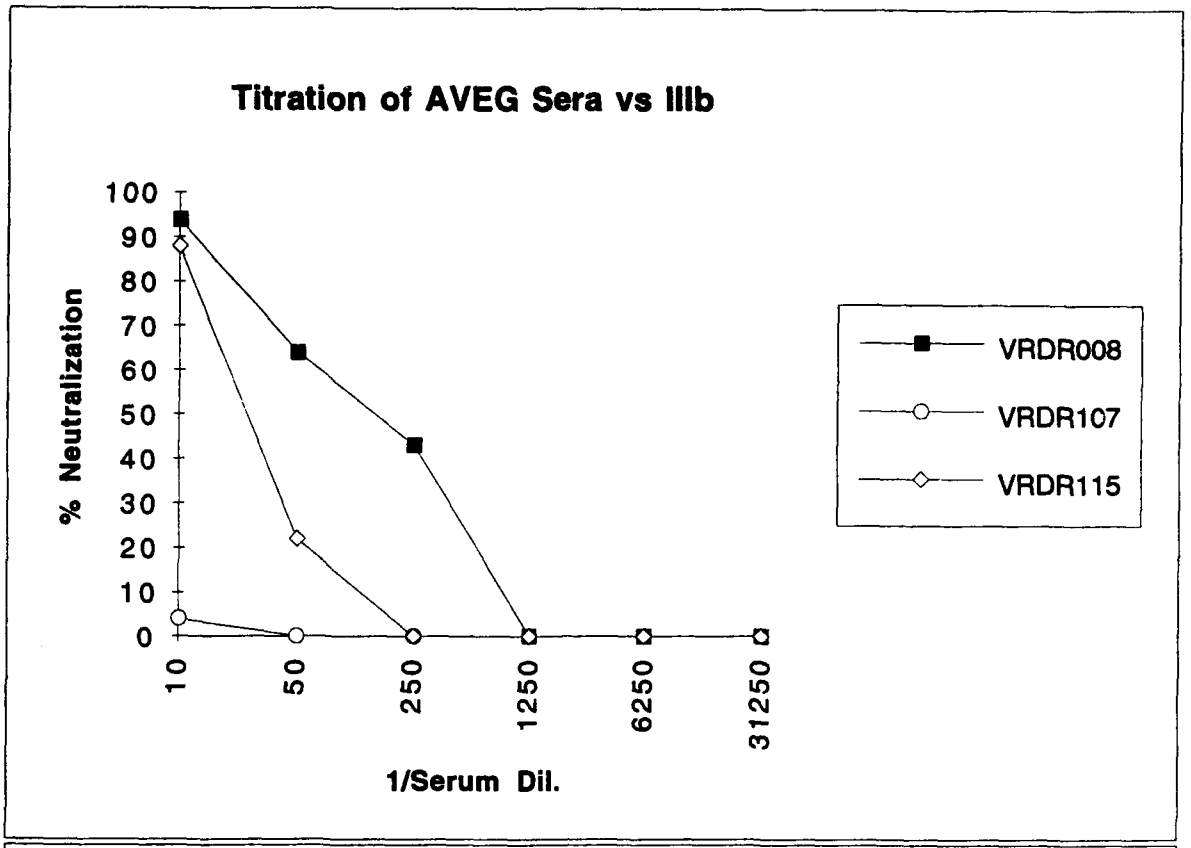

AVEG: IIIb vs Mn
% Neutralization
100
90
80
70
60
50
40
30
20
10
0
Pos 10828
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
Serum ID
IIIb
Mn

Figure 12


Titration of AVEG Sera vs IIIb
% Neutralization
100
90
80
70
60
50
40
30
20
10
0
10
50
250
1250
6250
31250
1/Serum Dil.
VRDR008
VRDR107
VRDR115

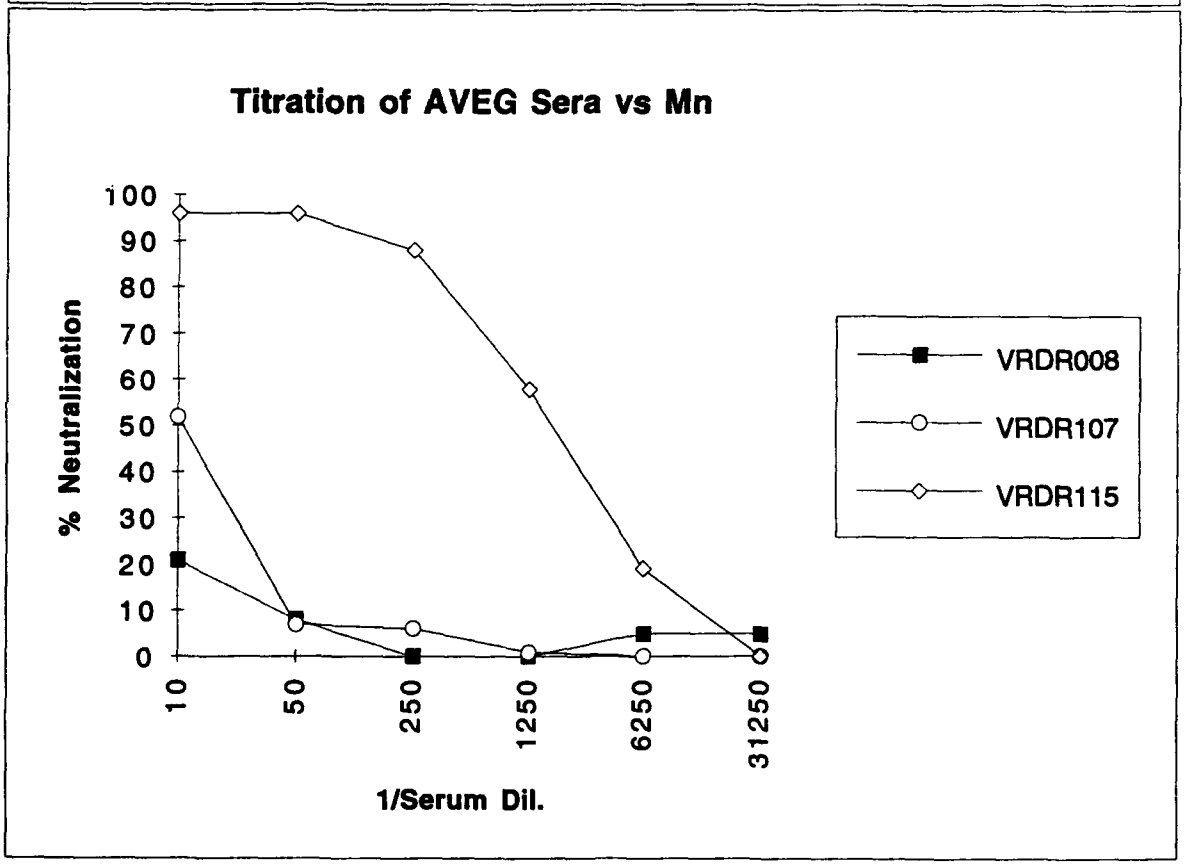
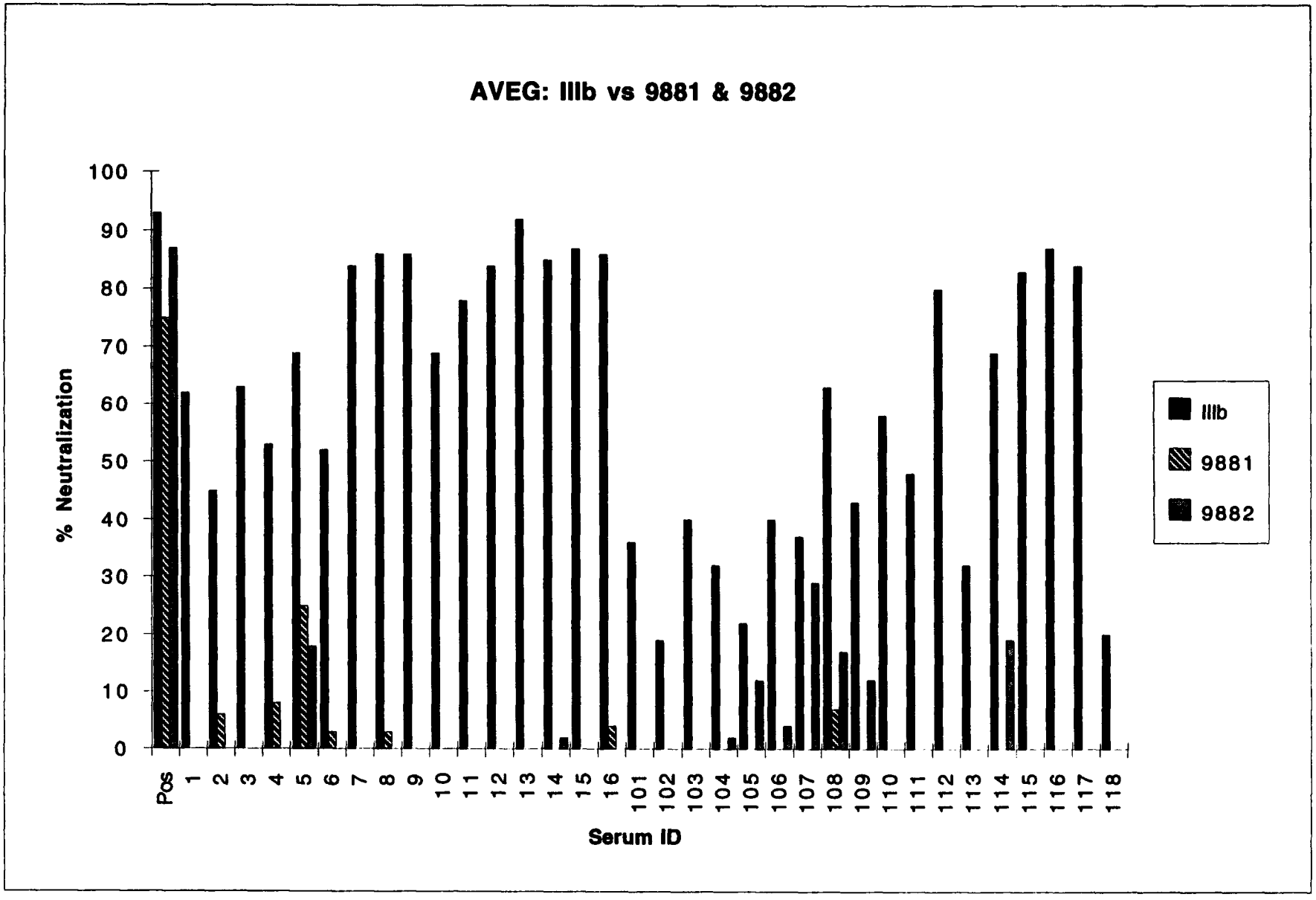

Titration of AVEG Sera vs Mn
% Neutralization
100
90
80
70
60
50
40
30
20
10
0
10
50
250
1250
6250
31250
1/Serum Dil.
VRDR008
VRDR107
VRDR115

Figure 13


AVEG: IIIb vs 9881 & 9882
% Neutralization
100
90
80
70
60
50
40
30
20
10
0
Pos
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
Serum ID
IIIb
9881
9882

laboratory strain Mn, even at very high virus inputs (4^{-2} dilution of virus stock). However, considerably longer incubation intervals are required for complete neutralization of the low passaged US1 clinical isolate 9881. These results have been confirmed in multiple experiments. Although far from complete, these initial studies suggest there is a marked difference in the neutralization kinetics of laboratory strains of HIV, such as Mn, and clinical isolates in the PBMC-based assay.

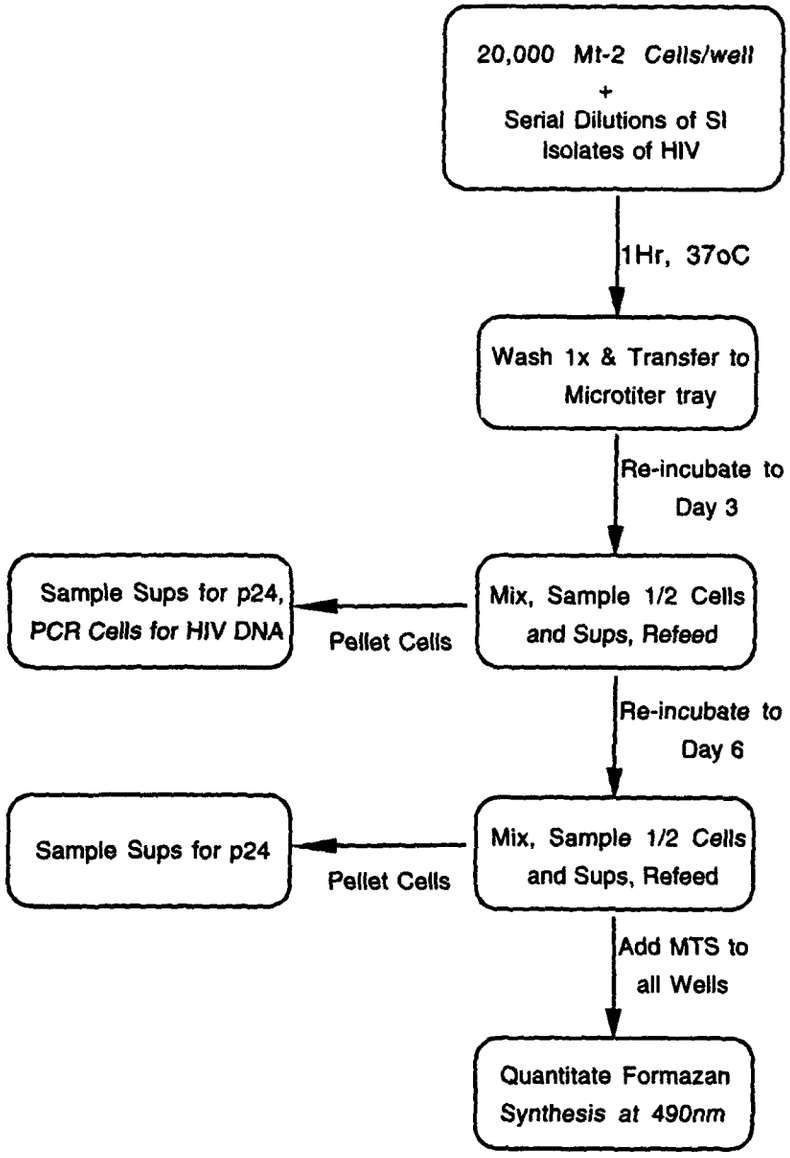
In collaboration with Dr. John Mascola of WRAIR, we have applied these optimized protocols to an evaluation of neutralizing antibodies in sera from HIV-seronegative vaccine volunteers obtained thru the AIDS Clinical Trials Network (part of an AIDS vaccine evaluation group; AVEG). Typical results are illustrated by the data in figures 11, 12 and 13. Figure 11 illustrates the results of a rapid screen of 34 sera using the IIIb and Mn labaratory strains of HIV. Three sera from this initial evaluation (8, 107 and 115) were selected to compare strain specificity and neutralization endpoints in a serum titration procedure.

The results of this titration are shown in Figure 12 and confirm the specificity of neutralization suggested by the initial screen (Figure 11). Serum 115 appears to have been generated by vaccination with Mn (50% neutralization or NT_{50} of ≈1250 for Mn vs ≈35 for IIIb); whereas, serum 8 appears to have greater specificity for IIIb than Mn (NT50 of ≈175 for IIIb vs <10 for Mn). On the other hand, serum 107 failed to react significantly with either virus.

At the request of NIAID we have extended these studies to determine whether vaccinees immunized with laboratory strains can neutralize low passaged clinical isolates of HIV. The data illustrated in Figure 13 indicates that the AVEG panel sera fail to neutralize at least two US clinical isolates, 9881 and 9882. A positive control serum (10828) known to neutralize most HIV strains did neutralize all three variants tested. These results have since been repeated using a number of geographically distinct isolates of HIV, none were neutralized by AVEG sera[15].

Figure 14

Procedure for Anaylsis of Antiviral Genes in Mt-2 Cells With SI Isolates of HIV



B. Development of In Vitro Assays for Evaluation of Antiviral Genes

Gene therapy for immunological disorders, cancers and a variety of infectious diseases is quickly becoming a reality. This approach has been expanded from "simple" gene replacement or augmentation therapy to correct a genetic defect (as in the case of adenosine deaminase, ADA, deficiency (16,17) to new genetic treatments for cancers (18) and infectious diseases such as AIDS (19,20). There have been numerous proposals for the treatment of HIV infections using antisense genes (21-25) and genes containing catalytic RNAs (ribozymes) (26,27). *In vitro* interference with viral replication has been accomplished by targeting gene constructs to viral structural proteins (28-32), components of HIV's regulatory circuits (33-35) and the virus receptor, CD4 (36). The number of antiviral gene constructs available for testing appears to be multiplying exponentially.

Preliminary *in vitro* evaluation of these therapies has been accomplished, for the most part, in artificial systems sometimes employing biochemical endpoints or in well established cell lines using laboratory strains of HIV. Little is known about the efficacy of such treatments for primary isolates of HIV in normal human peripheral blood mononuclear cells (PBMC) and there are no published reports of quantitative determinations of putative antiviral gene effects on primary isolate-induced cytopathogenesis. Moreover, the impact of these constructs on the differentiation and ultimate immune function of human bone marrow derived hematopoietic stem cells, the apparent conveyance of choice for some gene constructs, is little understood. Finally, there is no *in vitro* testing system available to bridge the gap between preclinical *in vitro* analyses and animal model systems such as the SIV model in macques.

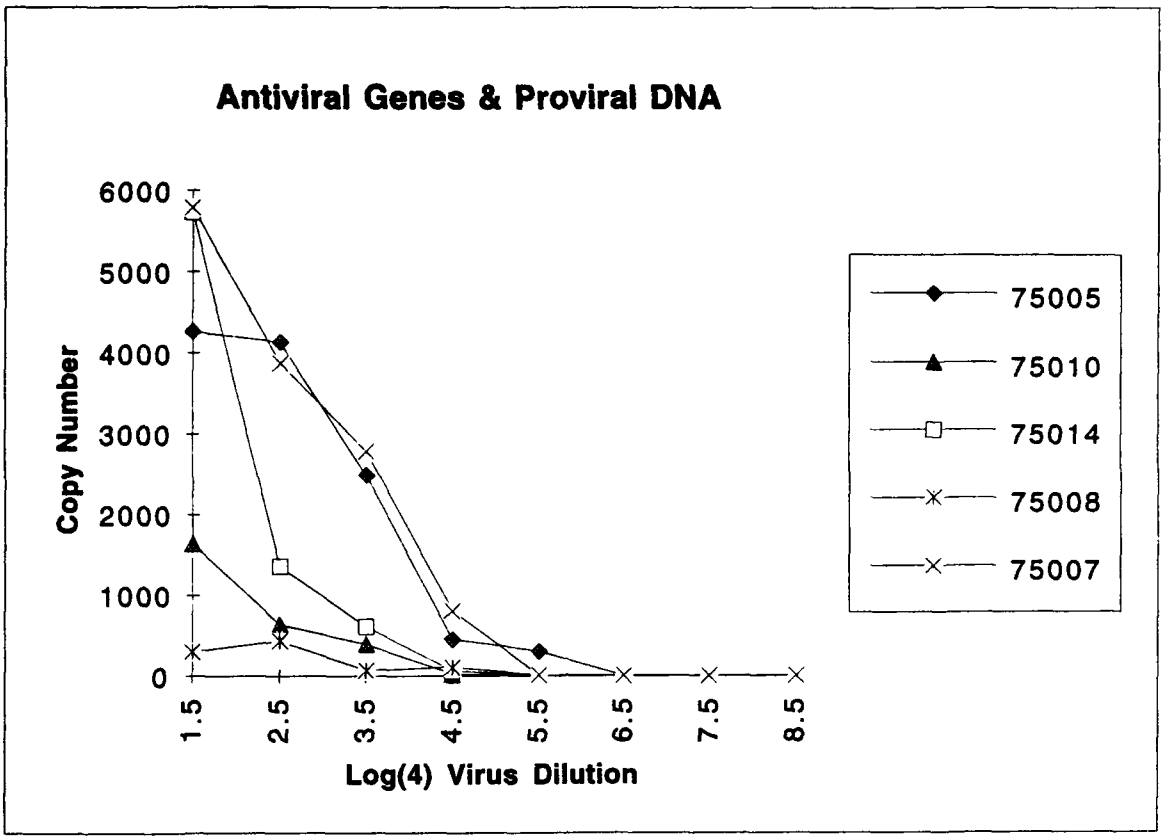
In support of a WRAIR collaboration with NIAID, SRA was requested to develop *in vitro* assay systems to assess the efficacy of antiviral gene constructs against low passage, clinical isolates of HIV. Initial studies were to involve the use of syncytial-inducing isolates of HIV in established cell lines previously transfected with antiviral genes. This is to be followed by similar studies in PBMCs that would permit evaluation of a broader range of clinical isolates or, eventually, a prospective patient's own cells. The cell line chosen for the preliminary studies was MT-2, a line that is productively infected with HTLV-1, but is sensitive to infection by ≈35% of patient isolates. Aliquots of these cells were sent to a number of laboratories in May of '93, transfected MT-2 cells were only received from 2 including Dr. Richard Morgan, at NIH and Dr. Joseph Mosca of the Henry M. Jackson Foundation.

A flow chart of the multiparameter procedure used for initial evaluation of antiviral gene constructs is illustrated in Figure 14. Three endpoints were utilized initially, these included supernatant p24, quantitative PCR for proviral DNA and a metabolic determination of viral cytopathic effects. However, of the three, only p24 and PCR for quantitation of proviral DNA proved to be reliable measures of clinical isolate replication and examples of those results provided in this report (Figures 15,16 and Table 5).

In the experiment from which Figure 15 was derived, multiple dilutions of an AZT sensitive SI (syncytial inducing) HIV isolate was evaluated in parental MT-2 cells plus seven MT-2 lines containing various gene constructions including (alpha IFN producers, 75007 and 75010, tat producer 75008 and four construct controls, 75005, 75012, 75013 and 75014). The results illustrated in the top panel of Figure 15 suggest that some antiviral activity, as determined by proviral DNA quantitation, may be present with one of the IFN producing lines, 75010. Similarly, there is also some suggestion that the tat producing line (75008) may possess antiviral activity. Production of tat, however, should lead to enhanced production of HIV by stimulation of viral mRNA production. The proviral DNA data are not supported by the p24 results in the bottom panel of Figure 15. No significant difference is seen in HIV production by control cell lines (975005, 75012, 75013 and 750140) and those containing putative antiviral genes (75007 and 75010).

Negative results were also obtained with a number of other viruses including a nef deleted variant of IIIb (LS0001) and the wildtype IIIb (Table 5). Poly-Tar constructions, also in MT-2 cells recieved from Dr. Richard Morgan of NIH, were analyzed in a separate study using HIV_{IIIb} as a target (Figure 16). In this analysis a positive control, the drug AZT, was included to insure that some level of protection was attainable in this system. Again, there was no apparent difference

Figure 15


Antiviral Genes & Proviral DNA
Copy Number
6000
5000
4000
3000
2000
1000
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
Log(4) Virus Dilution
75005
75010
75014
75008
75007

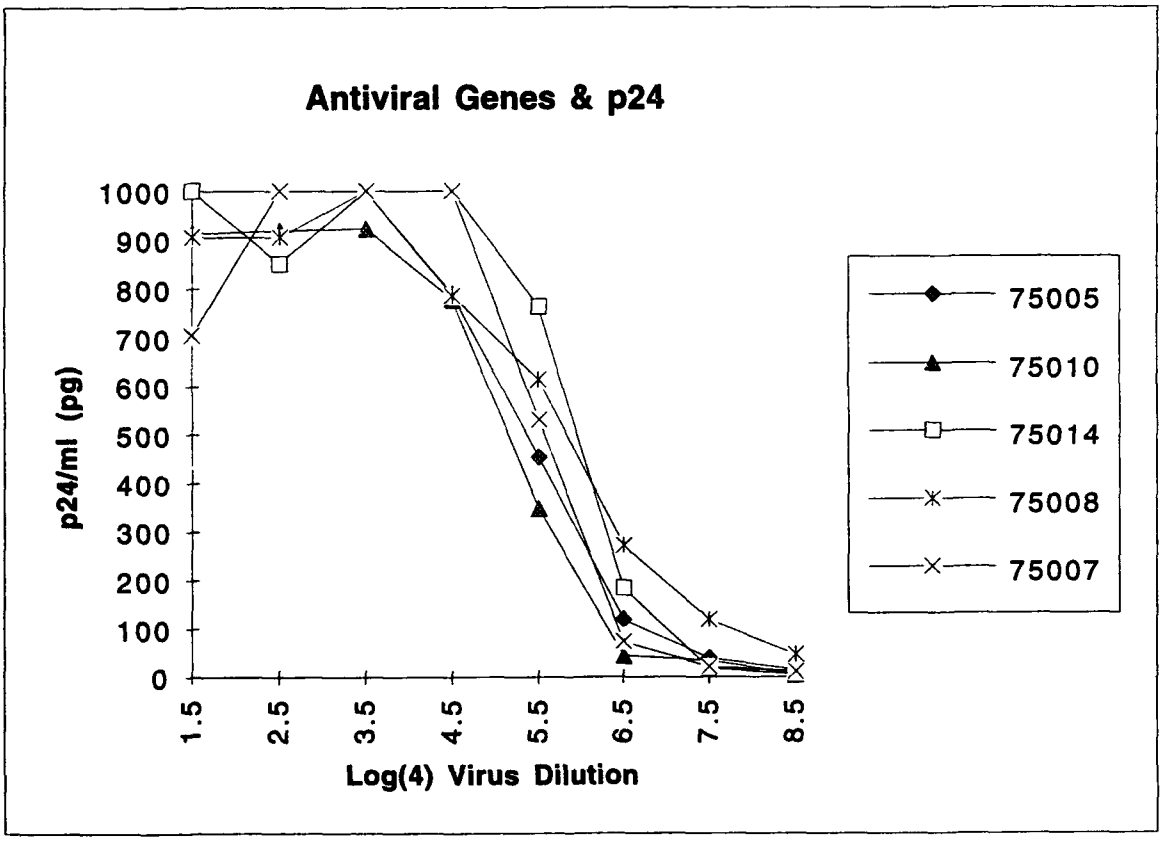

Antiviral Genes & p24
p24/ml (pg)
1000
900
800
700
600
500
400
300
200
100
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
Log(4) Virus Dilution
75005
75010
75014
75008
75007

TABLE 5

EFFECT OF INTERFERON ANTIVIRAL GENES ON REPLICATION OF HIV

	DILUTION OF VIRUS STOCKS								
Nef Deleted HIV IIIb	1.0E-5	3.16E-4	1.0E-4	3.16E-3	1.00E-03	3.16E-02	1.00E-02	3.16E-01	1.00E-01
750010	10.82*	15.73	40.36	158.20	211.00	649.60	3,189.00	1,761.00	>>>>>>>
	11.80	20.65	48.27	91.98	255.10	649.60	3,046.00	1,381.00	>>>>>>>
	6.89	32.47	40.36	88.98	283.00	641.80	3,400.00	762.80	>>>>>>>
750014	4.93	17.70	41.35	85.00	218.20	839.00	>>>>>>>>	>>>>>>>>	>>>>>>>
	1.99	18.68	39.38	90.98	498.90	616.20	3,081.00	>>>>>>>>	>>>>>>>
	9.84	23.60	53.22	118.00	351.80	976.30	>>>>>>>>	2,926.00	>>>>>>>
HIV IIIB.2									
750010	10.82	34.44	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>
	10.82	16.71	864.70	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>
	6.89	15.73	329.80	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>
750014	13.77	28.53	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>
	8.85	18.68	644.00	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>
	239.70	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>	>>>>>>>

* = picograms of p24; >>>>>>> = Too high to quantitate accurately, i.e. outside the standard curve

Figure 16

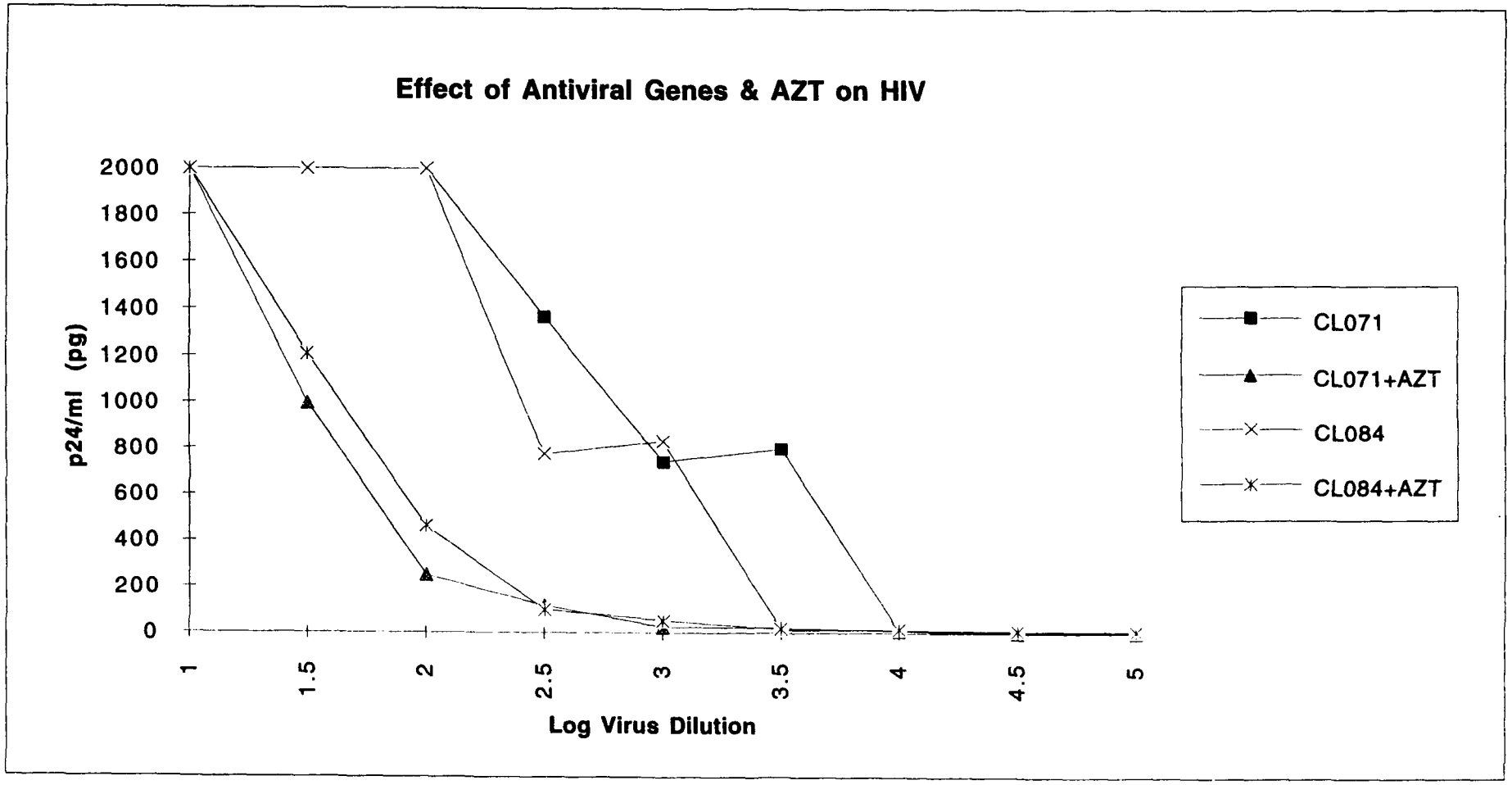
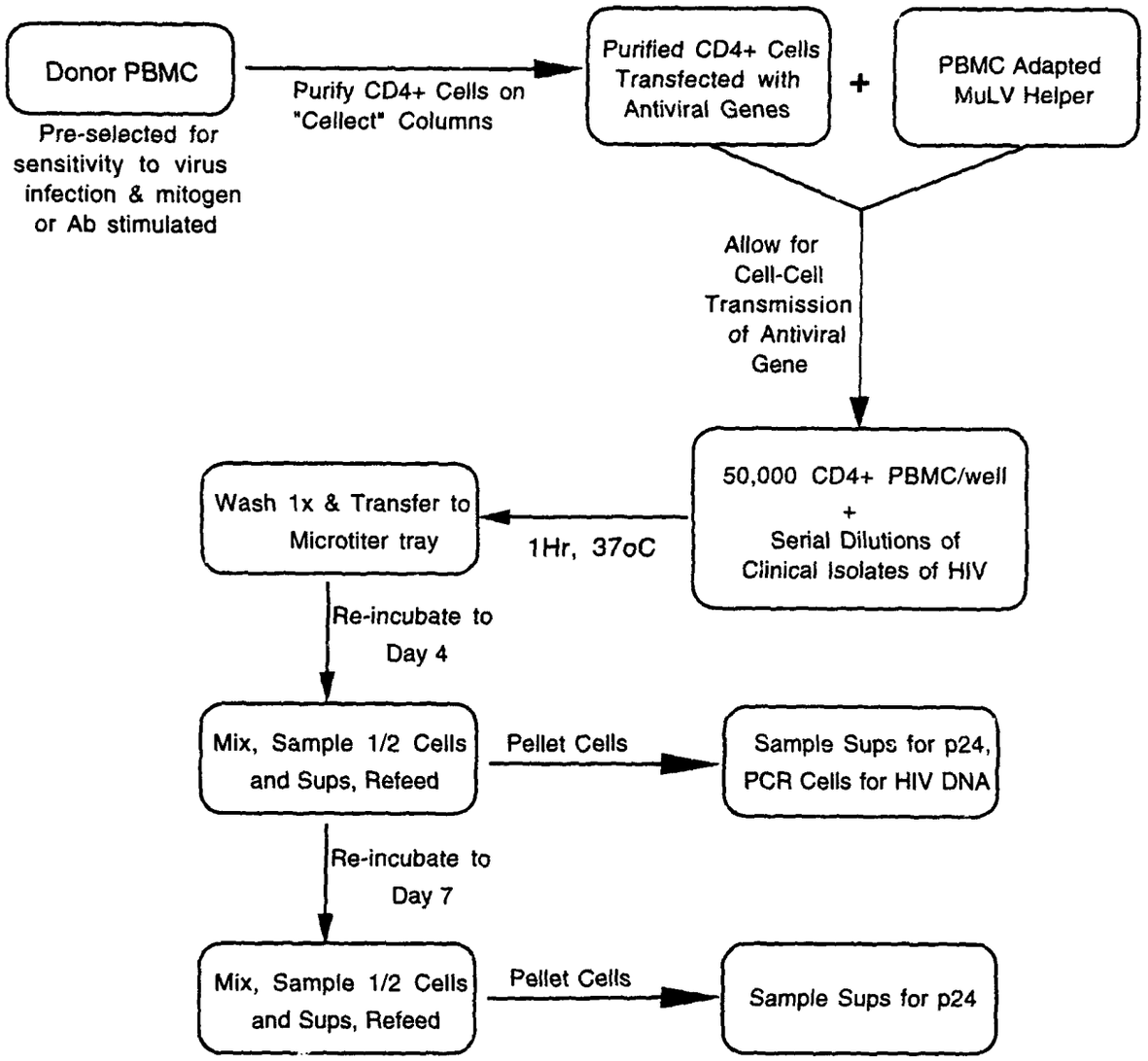

Effect of Antiviral Genes & AZT on HIV
p24/ml (pg)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
1.5
2
2.5
3
3.5
4
4.5
5
Log Virus Dilution
CL071
CL071+AZT
CL084
CL084+AZT

Figure 17

Potential Procedure for Anaylsis of Antiviral Genes in Purified CD4+ PBMC With Low Passage Clinical Isolates of HIV



between the construct control (CL071) and the poly-Tar gene construct containing line (CL084). However, in this instance the infection was clearly depressed by the presence of AZT.

The failure to demonstrate significant antiviral activity might have resulted from the production of HTLV-1 by the MT-2 cells. This virus could concievably interfer with expression, regulation or activities of the antivirals. New studies are underway to evaluate additional cell lines as potential antiviral gene carriers, e.g. MT-4, A3.01 and SupT-1 cells. In addition, we have begun to evaluate the possible use of purified CD4+ PBMCs in direct assay of these and other constructs using the protocol suggested in Figure 17. The first of these new studies will be availble in the early part of FY94.

C. Antiviral Drug Studies and Other Phenotypic Analyses

The primary responsibility of this group is to utilize an *in vitro*, peripheral blood mononuclear assay to determine the incidence and clinical significance of AZT resistance in patients with HIV disease being treated with AZT (RV43 study). During the period 10/92-10/93, this group also participated in the following additional studies:

1. RV65 - to determine the time course of development of resistance to experimental compound U-87201E in patients with HIV isolates demonstrating *in vitro* resistance to AZT.

2. CPCRA - prospective evaluation of the development of *in vitro* anti-retroviral resistance in HIV-1 isolates obtained from patients participating in the CPCRA Combination Nucleoside clinical trial.

3. The Johns Hopkins University/MACS studies.

4. The Johns Hopkins University seroconverter study.

5. The *in vitro* testing of experimental anti-retroviral compounds using HIV-1 isolates.

The drug sensitivity assays performed on RV43 and RV65 isolates resulted in the determination of the *in vitro* drug inhibitory concentration of four anti-viral agents for each virus isolate tested. An example of a final report for an RV43 patient is presented in Table 6. During this period 268 drug sensitivity assays were performed and reported for RV43 patient isolates. Assays performed on the six patients enrolled in the RV65 study examined the *in vitro* resistance to AZT, ddC, ddI, and

U87201E. Thirteen assays were performed before this study was terminated (Table 7).

For the additional studies, 187 CPCRA specimens were received and processed for virus isolation. It is anticipated that during the next fiscal year virus titration and drug sensitivity assays will be performed on these isolates. For the Johns Hopkins University MACS study the drug testing group received 52 vials of frozen cells from individual patients for virus isolation. Virus was isolated from 30 of these specimens and we were requested to determine the virus titration and *in vitro* resistance to AZT for 16 of these isolates (Table 8).

For the Johns Hopkins University seroconverter study, we received 16 isolates for testing *in vitro* AZT resistance. Because of low virus titration results obtained for two of these isolates, assays could only be performed on 14 of these specimens (Table 9).

Several experimental compounds were tested to determine their ability to inhibit replication of HIV isolates. These drugs were obtained from the laboratories of the Department of Applied Biochemistry of the Walter Reed Army Institute of Research, the Laboratory of Medicinal Chemistry at the National Cancer Institute, and private pharmaceutical companies. These compounds were tested by using isolates from RV43 patients and AZT-resistant and sensitive control virus isolates (Tables 10-14).

Table 6
Drug Testing Report

SRA #: 14168

Date Virus Titration Set-up: 8/24/93 SRA #: 14168 Titr.1

Titration Data

Assay Date	O.D. Cutoff	Number of + wells per Virus Dilution 16	64	256	1024	4096	16384	65536	$TCID_{50}$	4-Drug Plate Virus Stock Required
9/1/93	0.305	6	6	6	6	6	6	2	51991	0.080

Date Drug Sensitivity Set-up: 9/14/93 SRA #: 14168 4Drug.1

Date Drug Sensitivity Assayed: 9/23/93

AZT (uM)	$p24x10^5$	Fraction Affected	ddC (uM)	$p24x10^5$	Fraction Affected
0	1.06		0	1.06	
0.001	0.84		0.01	0.42	0.60
0.01	0.70		0.1	0.05	0.95
0.1	0.85	0.20	1.0	0	0.99
1.0	0.76	0.28			
5.0	0.23	0.78			

M = 0.6445
Dm = 1.3769
R = 0.8918

M = 0.9098
Dm = 0.0054
R = 0.9926

ddI (uM)	$p24x10^5$	Fraction Affected	XM323	$p24x10^5$	Fraction Affected
0	1.06		0	1.06	
0.1	0.99		0.01	0.82	0.23
1.0	0.81		0.03	0.22	0.79
5.0	0.84	0.21	0.1	0	0.99
10.0	0.60	0.43	1.0	0	
25.0	0.08	0.92			

M = 2.3738
Dm = 9.5796
R = 0.9852

M = 2.5235
Dm = 0.0167
R = 0.9989

Table 7

IC50 (uM) Results for RV 65 Patients

SRA #	Date Received	TITER	AZT	ddI	ddC	U8
15796	9/09/92	16384	1.6201	3.6327	0.0465	1.7871
16572	9/29/92	6472	3.9854	6.1778	0.0310	2.0545
15886	9/11/92	32768	0.1100	0.1660	0.0011	2.5790
16522	9/28/92	5184	1.4459	3.2107	0.0980	8.2969
18112	11/06/92	41476	0.1098	0.3493	0.0035	0.0010
19307	12/16/92	809	0.0264	--	--	0.0281
18601	11/24/92	12944	4.5192	14.6049	0.0229	0.0011
19595	12/30/92	25888	>5.000	>25.000	0.1483	>10.000
20287	01/27/93	65536	2.5910	15.7352	0.0788	4.1162
18153	11/09/92	32768	1.5339	3.7822	0.0913	0.0093
19423	12/18/92	65536	4.7991	14.9851	0.1204	1.9607
18161	11/09/92	16384	0.5127	1.7237	0.0146	0.0748
19592	12/30/92	809	0.1440	--	--	0.0865

Table 8

AZT IC50 Results for MACS Isolates

		IC50 (uM)
V10	20344	0.1519
V13	40743	2.3671
V10	20402	0.0036
V14	21148	TCID50 too low to perform test
V12	20742	TCID50 too low to perform test
V13	20275	0.0044
V13	10156	0.0212
V13	20208	0.8735
V13	20727	0.6990
V13	40043	0.0062
V13	40791	5.9997
V14	20482	TCID50 too low to perform test
V13	41102	0.0029
V14	20577	0.1589
V14	20111	TCID50 too low to perform test
V14	20576	0.4987

Table 9

AZT IC50 Results for JHU/Seroconverter Study

Hopkins #	AZT IC50 (um)
92536	0.0077
925737	0.0728
926026	0.0126
926028	0.0428
925563	0.0254
925715	0.0063
925720	0.0106
925730	0.0160
925750	0072
925773	0.0152
925933	0.0038
926289	0.0060
926291	0.0289
926293	0.0588

Table 10

IC50 Results for Experimental Compounds

Isolate	IC_{50} (uM) AZT	NEPA	5-aza cytidine	4 thio adenosine
A012 Sensitive	0.0213	0.0092	0.1211	0.0928
A012 Resistant	2.1345	0.0012	0.1733	0.6323
A018 Sensitive	0.0101	0.0122	0.1002	0.0932
A018 Resistant	1.2325	0.0008	0.8342	0.0645
18199	1.6456	0.0096	1.2378	0.2122
18190	0.0433	0.0045	0.2134	0.1074
18601	2.2337	0.0754	0.3113	0.2054

Table 11

IC50 Results for Experimental Compounds

	IC_{50} (uM)						
Isolate	AZT	PMEA	GEM	RND	DZNEP	DZA	DARI
18353	0.0051	0.8973	2.5481	4.9154	0.0126	1.1222	0.2854
18371	0.0134	0.4434	1.2783	9.4961	0.0097	0.1168	0.5526
18431	0.3514	1.3454	0.3835	1.1613	0.0004	0.0085	0.0106
18453	0.0031	0.4715	0.0822	1.9867	0.0021	0.0124	0.0078
18540	0.0082	0.3371	20.000	34.854	0.0044	6.3069	0.1055
18563	0.9711	6.8104	2.6721	2.8359	0.0302	0.3670	0.2137
18190	0.0356	1.0271	6.3246	17.5080	0.0011	0.1746	0.0587
18199	2.3802	5.5494	6.3246	11.5776	0.0139	>1.0000	0.1625
18202	0.0107	0.3185	12.0472	21.3283	0.0342	>1.0000	0.2704
18574	0.1302	0.9165	>20.0	>20.0	0.0834	>1.0000	>1.000
18601	2.1106	20.6217	>20.0	>20.0	0.0431	0.3364	0.3018
18678	0.0146	0.1030	1.8379	4.3243	0.0329	0.2542	0.4040

Table 12

IC50 Results for Experimental Compounds

IC_{50} (uM)

Isolate	AZT	DZNEP	DZA	DARI
A012 Sensitive	0.0143	0.0112	0.1654	0.1964
A012 Resistant	2.0865	0.0011	0.1108	0.3876
A018 Sensitive	0.0232	0.0129	0.2777	0.2322
A018 Resistant	2.2322	0.0065	0.6654	0.1012
18199	1.9877	0.0102	0.8988	0.1755
18190	0.0546	0.0087	0.1144	0.0674
18601	1.9987	0.0564	0.2213	0.1054
18431	0.4753	0.0021	0.0044	0.0088
18540	0.0100	0.0033	1.2322	0.0899

Table 13

IC50 Results for Experimental Compounds

IC_{50} (uM)

Isolate	AZT	323	GEM	RND
18190	0.0677	0.0281	0.9196	9.9673
18199	3.9365	0.0272	2.2892	4.1632
18202	0.0091	0.0258	6.3246	5.3919
18574	0.0066	0.0184	3.3031	4.2484
18601	5.2208	0.0339	>150	>20
A018R	1.6532	1.3234	2.1232	6.7854
A018S	0.0127	0.9342	2.7323	5.3745

Table 14

IC50 Results for Experimental Compounds

IC_{50} (uM)

Isolate	AZT	3182	3183	3208
A012 Sensitive	0.0212	3.4006	1.9615	3.1623
A012 Resistant	1.5485	4.0688	3.7096	3.1623
A018 Sensitive	0.0102	2.6825	1.6284	3.7096
A018 Resistant	1.3235	>5.0	1.8132	1.3722
014A	0.0232	2.0324	1.0188	3.1623
014B	2.1321	2.8943	1.6986	2.6957

3. Data Group Section

The computer capabilities of contract DAMD17-92-C-2504 took a major leap forward during FY93 with the installation of a wide-area network encompassing the Taft Court, Shady Grove, and Key West facilities of SRA. This involved several steps, including upgrading of the RLIMS database hardware, installation of linked local area networks (LANs), provision of 386-type or better computers for all personnel and provision of new software packages.

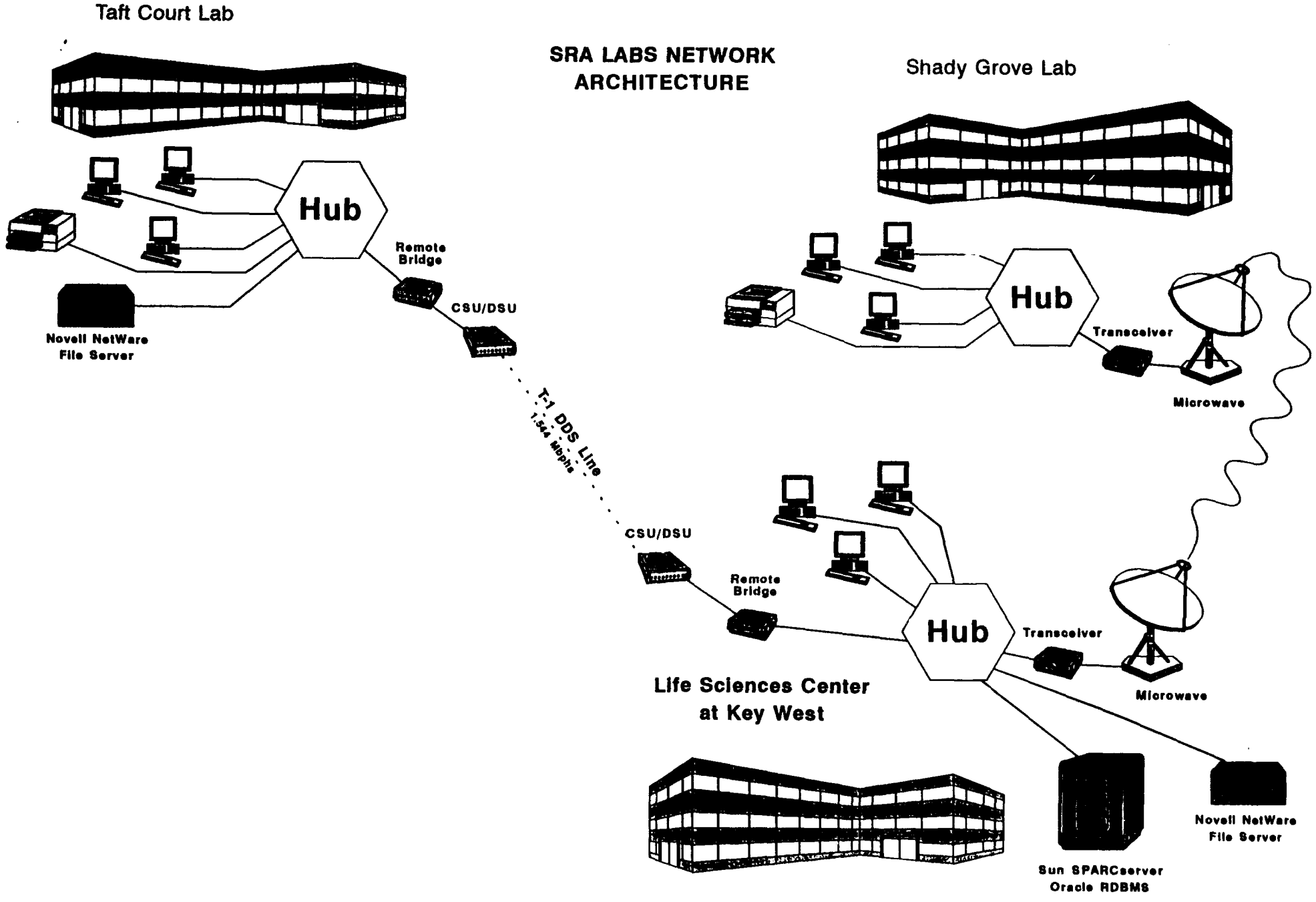
3.A. Establishment of Information System Hardware

The RLIMS database upgrade step involved replacement of an aging 386-based 8-user SCO Unix-based host with a Sun SPARCserver 670, capable of supporting upwards of 100 users and accommodating 5 years of data growth. The response time of queries on the system improved 1000% in many instances. The data support group was consolidated at the Key West facility, improving internal efficiency. Programming productivity has increased enormously due to the new high-speed equipment.

The second step involved linking the three sites internally with LANs and connecting those LANs via T-1 digital phone lines and microwave radio. Standard Windows-based applications were installed for universal access, including WordPerfect, Excel, and cc:Mail. Now, with the exception of the Macintosh, a user can perform any task from any workstation at any site. New computers were acquired for key personnel to replace 286 PCs, and eight computers were acquired for pooled use by lab technicians. The architecture of SRA's new laboratory information management system is illustrated schematically on the next page.

3.B. Introduction of New Software

1. Oracle Database - The Oracle database software is in the process (at end of FY1993) of being upgraded to from Oracle v6 to ORACLE7. This will improve data integrity and security and improve programming by enabling much of the data validation and processing logic to be coded into the database rather than the applications. SRA's Relational Laboratory Information Management System (RLIMS) utilizes Oracle and resides on a Sun SPARC server located at SRA's Key West Facility. The RLIMS Database contains patient identifiers, specimen information, freezer inventory, and results associated with the contract. The benefit of the RLIMS database being on the network is the ability for end users to access information concerning specimens they are assigned via Forms or Reports that reside on the RLIMS system.Investigation began into the acquisition of new graphical Windows/Mac front-end software to replace the character-mode Unix interface, and graphical


Taft Court Lab
SRA LABS NETWORK ARCHITECTURE
Shady Grove Lab
Hub
Remote Bridge
CSU/DSU
Novell NetWare File Server
T-1 DDS Line
1.544 Mbpts
Hub
Transceiver
Microwave
CSU/DSU
Remote Bridge
Hub
Transceiver
Microwave
Life Sciences Center at Key West
Sun SPARCserver
Oracle RDBMS
Novell NetWare File Server

end-user query software to enable scientists and lab technicians to build their own reports from the database without programming.

2. Excel Spreadsheet - Excel is the standard spreadsheet software package used on SRA's Lab Network. This package permits Lab supervisors as well as the technicians to analyze raw data produced by experimentally and stored in RLIMS. Currently raw data is either typed in or downloaded directly into a spreadsheet template for calculation or statistical evaluation. Once the spreadsheet is complete report formats including graphical charts can be applied and distrubuted through out the group via file sharing or our E-mail package CCMail.

3. CC Mail - This Software package allows contract personnel to communicate within their group and with others users on the network. This e-mail capability permits distribution of experimental protocols and data to all technical staff and interested parties without resorting to scheduled meetings. CCMail also supplies a central point for technicians to store messages and files associated with tasks assigned by the laboratory supervisors. The Data Group worked closely with various SRA contract personnel to create procedures and programs within RLIMS to handle experimental plate data for all experimental work. The Data Group wrote a number of reports, and formulated methods to e-mail RLIMS output to contract personnel in Excel format.

Finally, at the request of WRAIR personnel and the contract office's representative, a procedure was established to pass all contract generated data to WRAIR via the Henry M. Jackson Foundation's (HMJF) computer network. The first data transfer encompassed the period January 1988 through July 1993. HMJF will load the data into their Informix database for further processing at the discretion of WRAIR investigators. The data was transferred on 1/4" tape. Meetings were held to formulate plans for direct linking of the new SRA network with the WRAIR/HMJF computer network, allowing HMJF to access approved data downloads directly. HMJF agreed to procure and install the necessary equipment.

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Publications, Abstracts and Presentations

Below are included published reports, abstracts and presentations from work performed during the FY93 contract year by contract personnel (highlighted) often in collaboration with staff of the WRAIR and HMJF and other investigators.

1. Japour, A.J., Mayers, D.L., Johnson, V.A., Kuritzkes, D.R., Beckett, L.A., Arduino, **J., Lane**, J., Black, R.J., Reichelderfer, P.S., D'Aquila, R.T., Crumpacker, C.S., The RV-43 Study Group and The AIDS Clinical Trials Group Virology Committee Resistance Working Group, Standardized Peripheral Blood Mononuclear Cell Cultrue Assay for Determination of Drug Susceptibilities of Clinical Human Immunodeficiency Virus Type 1 Isolates. Antimicrobial Agents and Chemotherapy, 37(5):1095-1101, 1993.

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3. **Weislow, O.S., S. Belay, S. Snyder, M. Yeager**, F. McCutchan, J. Mascola and D.L. Mayers. Optimization of Peripheral Blood Mononuclear Cell (PBMC)-Based Neutralization Assays for Early Passsage Clinical Isolates of HIV-1. Sixth NCVDG Meeting, Conference on Advances in AIDS Vaccine Development, Washington D.C., 1993.

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5. Chiang, P.T., Joshi, B., Hewlett, I.K., **Lane, J.R.**, Doctor, B.P., Burke, D.S. and Mayers, D.L., 3-Deaza Adensosine Analogs as Novel Anti-HIV Drugs. for presentation at the

Amer. Soc. of Pharmacol. & Exptl. Therapeutics Meeting in San Francisco, CA July 30, 1993.

6. Mayers, D.L., Wagner, K.F., Chung, R.C.Y., **Lane, J.R.**, Vahey, M.T., **White, F.A.**, Ruiz, N.M., Hicks, C.B., **Weislow, O.S.**, Gardner, L.I., Burke, D.S. and the RV43 Study Group, Zidovudine (AZT) Resistance is Temporally Associated with Clinical Failure in Patients on AZT Therapy. for presentation at the First National Conf. on Human Retroviruses and Related Infections, Washington, D.C. Dec. 1993.

7. **White, F.A., Tran, M., Zhao, X., Antezana, M., Weislow, O.**, Vahey, M. and Mayers, D.L., An Improved 215 PCR Assay for DNA and RNA. presented at the Third Workshop on Viral Resistance Gaithersburg Hilton, Sept 19-23, 1993.